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THE CONCENTRATION OF HEMOGLOBIN IN SOME COLD WATER ARCTIC FISHES[1]

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The demonstration by Ruud in 1954 that several species of cold water antarctic fishes lack hemoglobin inspired us to check on the iron content in the blood of some arctic fishes, which live all year round at extremely low temperatures. As Ruud has pointed out, a low temperature would favor a low metabolic rate and would enable plasma and water to hold more oxygen, both factors probably essential for the evolution of a fish without hemoglobin. One may point out that, although the metabolic rate in the few cold water arctic fishes that have been investigated clearly indicates an adaptive elevation, it still falls far behind the rate of a tropical fish at its habitat temperature (Scholander, Flagg, Walters and Irving, '53).

Our fishes were taken in Hebron Fjord, northern Labrador, in August. At depths of 100 meters and lower the temperature stays around —1.73°C. all year round. At depths of 5–10 meters in the summer it may rise to some 4–6°C. In the summer the oxygen content of the water at 100 meters' depth was found to vary between 6.7 and 6.9 ml/l, corresponding to 80% saturation. Nearer the surface it was 100% or more. There are hydrographic reasons to expect that these waters are well aerated throughout the year (Nutt and Coachman, '56). Blood was taken from the fishes by heart puncture, and iron analyses were performed according to the method by Wong ('28).

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[1] Contribution No. 892 from the Woods Hole Oceanographic Institution. This work was carried out on the Blue Dolphin Labrador Expedition, 1954, under the auspices of the Arctic Institute of North America, with funds provided by the Office of Naval Research.

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Table 1 shows the iron content of the blood of 4 species of arctic fishes caught in Hebron Fjord. The values range from 8.5 mg % to 21 mg %, which would normally correspond to an oxygen capacity of 3.4 vol. % to 8.4 vol. %.

Hall and Gray ('29) give iron values from 15 species of shallow water marine teleosts. They run from 45.5 mg % in the bonito to 11.5 mg % in the sand-dab. Excluding the Scombridae and the menhaden, with their exceptionally high iron content, the remaining 10 species average 19.9 mg%. Compared to these shallow water fishes, a series of 12 deep sea species have a range from 24.9 mg % in *Sebastes* to 11.9 mg % in *Cottunculus*, with an average of 19.6 mg % (van Dam and Scholander, '53). In table 2 we have listed the fishes in these two series which have the lowest iron contents in the blood.

TABLE 1

Iron content in the blood of Arctic fishes

(Blood pooled from several specimens)

	DEPTH OF CATCH	IRON
	m	*mg %*
Liparis koefoedi	100	8.5
Gymnacanthus tricuspis	100	11.3
Gadus ogac	2–5	17.2
Myoxocephalus scorpius	2–5	21.0

TABLE 2

Iron content in the blood of some southern fishes with low hemoglobin content[1]

(averages)

	DEPTH RANGE	IRON
	m	*mg %*
Cottunculus thompsonii[2]	750–915	11.9
Coryphaenoides rupestris[2]	390–865	11.9
Lophius piscatorius[3]	10–720	14.7
Opsanus tau[3]	1–50	13.5
Lophopsetta maculata[3]	1–70	11.5

[1] Depth ranges kindly furnished by Mr. W. C. Schroeder, Woods Hole Oceanographic Institution.

[2] van Dam and Scholander ('53).

[3] Hall and Gray ('29).

A glance at tables 1 and 2 will show that low hemoglobin content may be found among cold water arctic fishes as well as among cold water deep sea fishes of more southern latitudes, and shallow, relatively warm water forms, such as the sand-dab *(Lophopsetta)* are also represented. Hall and Gray ('29) pointed out that the more active species in their series, especially the Scombridae, had the highest iron values, whereas the sluggish, sedentary forms tended to be the lowest on the scale. There are, of course, many exceptions to this over-all rule. In our material *Gymnacanthus, Myoxocephalus,* and *Cottunculus* are all sculpins and presumably very similar in habits, yet *Myoxocephalus* has nearly twice as much blood pigment as the others, and while *Liparis* may be somewhat sedentary, as indicated by the suction cups on its pelvic fins, *Coryphaenoides* is certainly not.

The oxygen capacity of the blood of *Liparis* and *Gymnacanthus* at 15°–20° was found to average 3.7 and 4.1 volume per cent. Their plasma at — 1.7° could carry one more volume per cent dissolved oxygen. Even this small amount of hemoglobin represents, therefore, no less than a fivefold increase of the oxygen transport capacity over plasma alone. Whereas a 20° rise in temperature would almost halve the oxygen carrying capacity in a fish without hemoglobin, the *Liparis* or sand-dab would suffer only 10% reduction. Consequently, compared to our northern low-pigment fishes, the antarctic pigment-free fishes are much more severely restricted by temperature. One would suspect that pigment-free fishes, even in ice water, would have developed some compensatory means to offset the handicap of the low oxygen carrying capacity of their blood.

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SUPERCOOLING AND OSMOREGULATION IN ARCTIC FISH[1]

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Department of Zoology

FIVE FIGURES

It is generally recognized that the freezing point of the blood of most teleost fishes is around — 0.5 and — 0.8°C. (cp. Black, '51). It is also known that Eskimos in many places catch fish through the sea ice, sometimes in the middle of the winter when the water temperature is — 1.7° or colder. Hydrographic work during the summer by Iselin ('32) and Nutt ('53) established that the water at the bottom of several fjords in northern Labrador maintains a temperature of — 1.7° to — 1.8°C.; i.e., right at the freezing point of the fjord water. By dredging the bottom with a trawl, R. H. Backus brought to light a considerable fish fauna living at these extreme temperatures ('51, '53).

The present investigation originated with a simple question directed to the senior author by Dr. R. H. Backus: "When arctic fishes swim about in ice water at — 1.7° to — 1.8°, why don't they freeze? Do they have twice as high an osmotic concentration as ordinary fishes, or what is the story?" Since the problem appeared attractively simple, it was decided to make a quick dash to Nain in northern Labrador in April 1953, and return with the answer, which was easy to guess anyway. A wait of three weeks, while rain rotted the ice along the Labrador coast and made airplane landings impossible, diverted the expedition from Nain to the U. S. Air Force Base at Frobisher Bay in Baffinland. With the aid of Eskimos and dogteams two of the authors (Scholander and Hammel) managed to catch three tiny sculpins in the course of two weeks and at the cost of chopping a dozen holes through four foot ice. The largest fish (about 20 gm) yielded half a cubic centimeter of blood, the medium-sized fish was eaten by a dog, and the little one (about 5 gm) yielded a tiny drop of eye chamber fluid. Freezing point determinations made in a tent 20 miles from the base showed that these fish avoid freezing simply by becoming isotonic with the sea water.

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[1] Contribution no. 891 from the Woods Hole Oceanographic Institution. This work was carried out, in part on the Blue Dolphin Labrador Expedition, 1954, under the auspices of the Arctic Institute of North America, with funds provided by the Office of Naval Research.

These very clear but scant data could not go unchecked, so it was decided to obtain further data from the bottom of Hebron Fjord in northern Labrador, where the temperature stays near — 1.7° all year round. This led to a summer expedition in 1954 on the Blue Dolphin, under command of D. C. Nutt. To our bewilderment it was found that all the bottom fishes had a plasma freezing point of only — 0.9° to — 1.0°, and hence were permanently supercooled by almost one degree. Fishes caught in the warm surface water had very nearly the same values, and froze instantly in a vat of freezing sea water at —1.7°. When they were lowered in a net to exactly the same temperature at the bottom of the fjord they did not freeze. Conversely, when the bottom fishes, who lived all their lives at —1.7°, were put in freezing sea water of this temperature on the surface they froze. After all the thermometers had been rechecked, these conflicting facts had to be reconciled.

A chance observation on supercooled *Fundulus* at the laboratory in Woods Hole offered a solution to the mystery of the arctic fishes, and prompted a third expedition to check on the Frobisher Bay data. This took two of the authors (Scholander and Kanwisher) back to Hebron Fjord in March 1955 where fishes were readily obtained through the ice. At last, then, with two winter expeditions and one summer expedition behind us, we were able to answer this simple question of why the arctic fishes don't freeze while swimming in water of —1.7° to —1.8°.

Field facilities

The work in Baffinland (April 23–May 5, 1953) was conducted from the U. S. Air Force Base at Frobisher Bay. The field work was greatly assisted by the cooperation of the Royal Canadian Mounted Police stationed at the base. For the summer work in Hebron Fjord (July 8 to August 15, 1954), a small prefabricated and prefurnished plywood laboratory (8 × 12 feet) was set up on the shore near an area of cold deep water. The location was 17 miles inland from the Moravian Mission at Hebron. Ice for making cold mixtures and for other laboratory and camping needs was taken from a large snow field next to the camp and later from drifting ice in the fjord. Stirring motors and all other electrical equipment were run with 6 volt Hot Shot batteries. The winter work at Hebron (March 13–24, 1955) was made possible through the cooperation of the Reverend F. C. P. Grubb of the Moravian Mission, who generously provided us with native help, working space, and quarters.

In the summer the shallow water fishes were caught by gill net or hand line at the camp site, and the deep fjord material was obtained by dredging the bottom with a 16 foot regular shrimp try net (Marinovich Trawl Company, Biloxi, Mississippi). The trawl was operated with a U. S. Navy Bureau of Aeronautics light metal target-towing winch powered by a 2½ horse gasoline engine, and mounted in a 30 foot Newfoundland motorboat, run by Mr. Peter Hay, of Nain, Labrador. In the winter, fishes were obtained by jigging and baited traps.

Freezing point determinations

Determination of the freezing point, i.e., the temperature at which a small ice crystal is in equilibrium with a large amount of unfrozen solution, was made with the method of Pounder and Masson ('34), modified to fit field conditions. In this method freezing and melting are directly observed under magnification.

Apparatus (fig. 1). A 4–5 liter white enameled tin can, set into the lid of an insulated paraffin-coated plywood box, served as a cold bath. Mounted on the lid of the box was a low power binocular scope. The thermometer and sample tube were held next to each other by means of a friction clip, hinged to permit adjustment. A Magnatorque 6 volt miniature motor, mounted on the box, drove a propeller shaft directly from the armature to provide vigorous stirring. A flashlight bulb provided light. A plastic sheet painted black and white, when properly adjusted under the sample tube, made a good contrasting background against which the crystals could be observed. It was mounted on a wire stuck into a movable block on the lid of the box. The thermometer, graded in tenths of a degree, was calibrated for zero in a slush of clean snow and distilled water, kept in a Thermos bottle. The sample tube was made from a thin-walled 1.5–2 mm glass tubing pulled out to a long taper point. The open wide end was

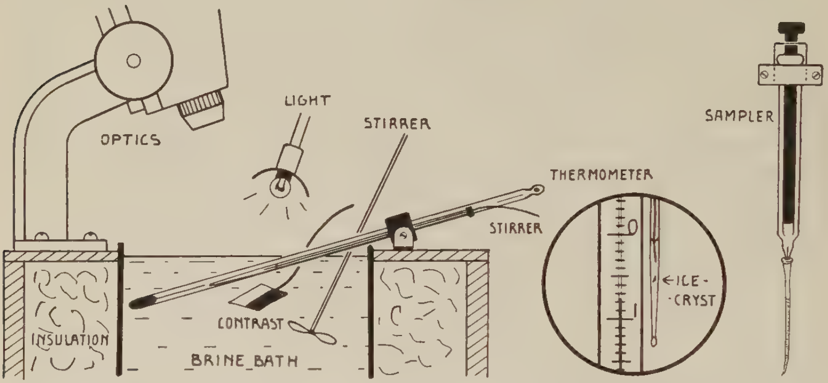

Fig. 1 Method for freezing point determinations modified for field work from the method of Pounder and Masson ('34). In circle: Thermometer and freezing tube. To the right: Syringe burette used as sampler.

closed by a vaccine stopper through which a Saran filament fitted loosely to act as a stirrer.

The temperature of the brine bath would rise 0.1° in three minutes, depending upon the room or tent temperature. It could be lowered or raised at any time by adding pieces of ice or snow, or by introducing hot water either directly or enclosed in a tube.

Procedure. The sample, 10 mm^3 or less, was drawn into a fine polyethylene tubing attached to a tuberculin syringe with a pulled out tip (fig. 1), and transferred to the sample tube, avoiding evaporation loss through exposure to the open air. It was shaken down into the tip of the sample tube, which was closed with the stopper and provided with the Saran filament for stirring. The sample was frozen in a Thermos bottle containing a snow and salt mixture at about — 10°. The sample tube was then placed in the friction clip and introduced into the brine bath, which was set at a few tenths of a degree lower than the freezing point of the sample. A test tube with warm water, or a finger, was held next to the stirred sample for a moment until all but a few crystals were melted. Stirring is necessary to abolish the concentration gradients produced by the freezing or thawing. As the temperature rose slowly in the brine bath, the ice crystals were watched. In melting, the edges of the crystals dull, and very small crystals disappear. This is the melting temperature. Before the last crystal was gone, the temperature was lowered slowly by adding snow to the brine. In freezing, the crystal edges sharpen up and new spicules may begin to form. This is the incipient freezing temperature. The average of these two readings is the freezing point.

Accuracy. In simple salt or sugar solutions melting and freezing temperatures were found to differ by some 0.05°, although theoretically they are, of course, the same. The average of these two readings gave the freezing point of NaCl, NH_4Cl and sucrose to within 0.05° of the Handbook values for $\frac{1}{2}$ and $\frac{1}{4}$ molar solutions, which covered the relevant range.

In fish plasma, especially that of the winter fishes, the melting and freezing temperatures were less sharp, and often as far apart as 0.1°.

Chloride determinations in blood plasma

Blood obtained by heart puncture was drawn into a syringe which had been rinsed with 0.2 M saline, and transferred to a dry vial containing a trace of powdered heparin. The plasma was separated in a hand centrifuge and stored for analysis. A sample of 0.1 ml of the plasma was digested on waterbath with a mixture of $AgNO_3$, H_2O_2 and nitric acid. It was titrated under ether according to the method of Rehberg as modified by Schnohr ('34). A 1 ml tuberculin syringe with plastic tip and friction clip (fig. 1) served as an excellent field micro burette. The titration and stirring were either performed free hand (Scholander, '47), or by screw activation and air bubbling (van Dam, '35). The plunger setting was read with a handlens and in either case duplicate analyses agreed within 0.01 M.

TABLE 1

Plasma freezing point of shore fishes in Hebron Fjord. Summer. Water temperature + 4° to + 7°. Depth 2–6 m

Gadus ogac, Fjord cod	— 0.77	— 0.77	— 0.78	— 0.81	— 0.83	— 0.85	(av. — 0.80)
Myoxocephalus scorpius, Sculpin	— 0.78	— 0.78	— 0.79	— 0.79	— 0.81	— 0.87	(av. — 0.80)
Salvelinus alpinus, Arctic char	— 0.80	— 0.80	— 0.81	— 0.82			(av. — 0.81)

Freezing point of plasma in summer fishes

Shallow water fishes. The freezing point depression of the plasma in fishes taken along the shore in shallow and relatively warm water will be seen in table 1. In all three species (fjord cod, sculpin, and arctic char) the freezing point usually lies between —0.75° and —0.85°, which is well within the limits of common southern marine teleosts (Black, '51).

Deep water fishes. The plasma freezing point of five species of fishes caught in the cold bottom water of the fjord at —1.73° is slightly lower (table 2), with specific averages running from —0.90° in *Liparis* to —1.0° in *Boreogadus.* The blood data were confirmed by freezing point determinations in the eye fluids and lenses of several of the same species of bottom fishes (table 3).

TABLE 2

Plasma freezing point of deep water fishes in Hebron Fjord
Water temperature — 1.73°

SPECIES	100 m			200 m	
Boreogadus saida	— 0.97			— 1.07	
Lycodes turneri	— 0.88	— 0.98		— 1.03	— 0.97
Liparis koefoedi	— 0.91	— 0.91		— 0.90	
Gymnacanthus tricuspis	— 0.94	— 0.95	— 0.89		
Icelus spatula	— 0.96				

TABLE 3

Freezing point of eye constituents in deep water fishes

SPECIES	100 m			200 m
Myoxocephalus scorpius	— 0.75[1]			
Lycodes turneri	— 0.91[1]	— 0.95[2]	— 0.96[2]	
Liparis koefoedi				— 0.93[2]
Gymnacanthus tricuspis	— 0.84[1]	— 0.85[1]		
Icelus spatula	— 0.84[1]			— 0.90[2]

[1] Chamber fluid.
[2] Lens.

The remarkable thing is that these fishes are living in water which is 0.75° to 0.85° below the freezing point of their blood. In other words, the whole fish fauna is supercooled by almost one degree. Inasmuch as the water temperature at the bottom stays constant all year at — 1.73° to — 1.75°, there is no reason to believe that these fishes change in the winter. Consequently they appear to live in a supercooled state all year round. It was repeatedly established that even large specimens of cod and sculpin had a body temperature within 0.1° of the sea water.

Stability of supercooled fishes

If the whole fish fauna at the bottom of Hebron Fjord is supercooled, the question arises as to how stable are such fishes against freezing. Several experiments to clarify this situation were performed both at the shore laboratory on Hebron Fjord and at the laboratory in Woods Hole.

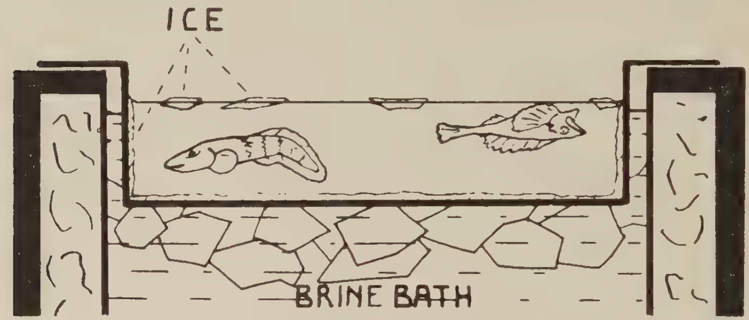

Fig. 2 Bottom fishes *(Lycodes* and *Gymnacanthus)* freezing in ice trough at — 1.7°.

(a) Freezing of bottom fishes at the surface. Many of the specimens caught in the trawl at a depth of 100 meters were lively and in good condition when brought up. They were put in a metal trough suspended in a cold mixture (fig. 2). By freezing out ice from the relatively brackish surface water, the temperature could be lowered to —1.7°, or further. When the bottom fishes were put into this water it was found that most of them became severely damaged. *Lycodes* froze partially, developing white patches and kinks in the tail. *Liparis* went into convulsions and got a kink in the tail. *Icelus* was stiff in rigor with gill covers expanded. Only some tomcods *(Boreogadus)* seemed to be unhurt at —1.73°, but they died with ice spicules visible in the eyes at —2.5°. *Lycodes* was killed at —1.9°.

(b) Freezing of shore fishes. In another series nine little sculpins (*Myoxocephalus*), of 10–15 gm weight, were caught by hand in the shallow water along the shore. These were put in the freezing trough. At —1° they were motionless on their backs, and ice masses filled their eyes. This could easily be seen under the binocular. With a touch of the fingers the eyes cleared. A medium sized sculpin, of 25–40 gm, froze stiff and white at —1.5° in 20 minutes, and died.

Four little sculpins were put in the ice trough at —1.7°. The eyes froze in a few minutes. One fish survived after 10 minutes in the bath. None survived after 20 minutes. Hence it was established that these little sculpins would regularly die of freezing when subjected to —1.7° in the trough for 20 minutes.

(c) Survival of fishes at —1.7° on the bottom. In two experiments sixteen small sculpins were put in a net and lowered to a temperature of —1.73° at 100 meters' depth. They were left there for one to three hours. When hauled up, fourteen were still lively. When some of these were put into the freezing trough at —1° their eyes froze in an hour.

Finally, in two experiments, two fjord cod and two sculpins were kept for four and eight days in the cold bottom water, without freezing or other signs of damage. They did, however, markedly lower the freezing point of their blood (see below).

(d) Hydrostatic effect on freezing point of plasma. Why then did the fishes not freeze at all on the bottom when they froze to death at the same temperature on the surface? One rather remote possibility was that the ten atmospheres' hydrostatic pressure would significantly lower the freezing point and protect the deep sea fish. This was tested by sealing a thin-walled freezing tube to a screw-activated piston pump. The air pressure was increased to ten atmospheres, and the freezing and melting were observed as usual. In plasma and eye fluid of sculpin and in isotonic saline solution the freezing points at 10 atmospheres were found to be 0.04°, 0.06°, and

0.08° lower than at 1 atmosphere, which checks the Handbook data for distilled water within the limits of our method. The pressure effect is therefore normal and nowhere near large enough to account for the non-freezing at the bottom.

(e) Stability of plasma supercooled to —2°. Plasma from *Myoxocephalus, Gadus ogac, Icelus,* and *Liparis* was transferred to the thin-walled freezing tubes. These were put into a brine solution in a Thermos flask, and placed in an icebox. Throughout most of a week's time the temperature was kept at —2°C. In a week most of the samples had become infected, but none froze. Dorsey ('48) kept vials of supercooled water for 10 months at —8° to —10° without the occurrence of freezing, so the results of our experiment were not surprising.

Ice seeding of supercooled fiishes

Sea water can readily be supercooled to —3° in a beaker suspended in a thermoregulated bath. It may be stirred gently by air bubbling. Several species of fish taken in cold water at Woods Hole in the winter (*Fundulus,* cunner, eel, toadfish, tautog) were found to survive for some time in such water. Cunner and *Fundulus* were kept in —3° water for 12 hours. They were torpid, but breathed slowly, and survived apparently without any harmful effects.

If the water containing the fish is supercooled to —2° or —3°, while gently stirred by air bubbling, and is then seeded with a few ice crystals, the water fills up with a whole flurry of ice crystals immediately. The practically motionless fish goes into a few violent convulsions, whitens in patches, and freezes stiff (fig. 3A). In other words, direct ice contact seeds the supercooled fish right through the integument. This happened in all of the five species enumerated above, and also in frogs.

If a fish frozen in this manner is plunged into sea water of room temperature, it will initially float due to the ice in its body, but will soon settle to the bottom when the ice thaws.

If such a partly thawed fish, which has ice only in its core (fig. 3C), is placed in a beaker of supercooled water, the water will shortly thereafter freeze, showing that the ice formation will also be propagated across the integument from inside outward.

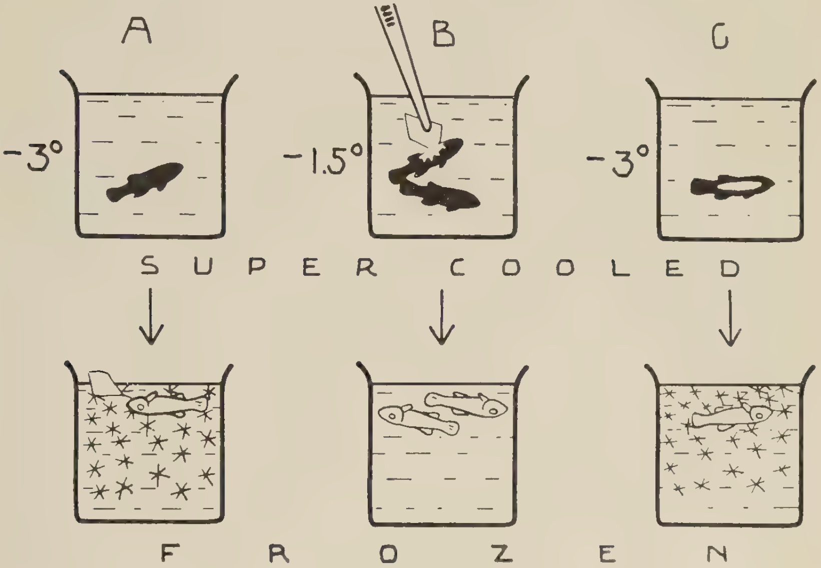

Fig. 3 Seeding experiments on *Fundulus*. A. Fish freezes when supercooled water is seeded. B. Supercooled fish freezes when touched with ice in water above freezing point. One fish may by contact seed another. C. Thawed fish with ice core freezes, and seeds supercooled water.

Fundulus, toadfish, and eel were supercooled in water of —1.5° to —1.7°; i.e.; in sea water warmer than its freezing point. If these fishes were touched with an ice cube (fig. 3B), they would go into convulsions, stiffen, and gradually whiten in freezing. Indeed, if only one fish were seeded and frozen, soon the other would freeze also by contact with the first, although the water could not freeze.

From the ice seeding experiments in live fishes it was clear that ice formation would pass right through the integument. This was studied further on pieces of skin of the eel, tautog, toadfish, and frog. The skin was stretched across one orifice of a piece of tubing, which was then filled with isotonic saline, and the whole assembly was placed in supercooled sea water. Seeding on either side of the skin would propagate through and seed the other side.

It was found that ice would propagate through a Corning glass filter, UF with 1–2 μ pores, but not through dialyzing cellophane at a supercooling temperature of —3°. In respect to ice propagation, therefore, fish and frog integuments seem to be relatively open structures. Considering that they maintain considerable osmotic gradients, it appears that the ice actually spears its way through the tissue. This is suggested by the following observation. If a fish is placed in freezing sea water with its gills exposed these will freeze white rapidly. If the fish is thawed and then put back in the cold water, the gills will not freeze again, probably because the ice crystals have pierced and damaged the osmotic barriers.

Freezing point of arctic fishes in the winter

From Dorsey's experiment with supercooled water samples, kept at —8° to —10° for months, it is clear that supercooled water possesses a certain degree of stability, and evidently more the less it is supercooled. Therefore, if ice seeding is absent, freezing may never occur at a slight degree of supercooling. This is undoubtedly what saves the supercooled bottom fishes in Hebron Fjord from freezing. Their supercooling is slight, and they live so deep that ice can never reach them.

But what about the shallow water fishes living all winter long near or amongst the floating ice? The answer was already indicated by our two little sculpins caught through the ice in Baffinland in the winter of 1953. Their plasma freezing point was —1.5° to —1.6°, i.e., very nearly the same as the sea water, and hence they could not be seeded by ice. This called for further study, and in March, 1955, we (Scholander and Kanwisher) returned to Hebron Fjord. Our Eskimo friends patiently jigged fjord cod and sculpins through holes cut in four foot ice along the shore in from 2 to 8 meters' depth of water. The fish were immediately taken into a heated tent next to the fishing hole, so that blood could be taken by heart puncture. Outside, the blood froze in the needle immediately.

The results will be seen in table 4. The sea water was at its freezing point of —1.73°, and the plasma freezing point in the cod averaged —1.47°, in the sculpin —1.50°, as against the summer value of —0.80° for both species (fig. 4).

The shallow water winter fishes had therefore almost doubled their osmolarity, but some were nevertheless as much as 0.2–0.4° supercooled. Several specimens were therefore transferred directly into a large freezing trough which contained floating pieces of ice in sea water. In this, most of the fishes died, undoubtedly by contact freezing. Two codfish were placed in a flour sack together with a piece of clear ice, and were suspended in the fjord. After a day one fish died, very probably from freezing. The other stayed alive, and was taken out of the bag, still living, after three days. It had then a freezing point of — 1.52° and a markedly elevated chloride concentration (cp. fig. 5).

We see, therefore, that considerable adjustments take place in those fishes which risk seeding by contact with ice. From the experiments where sculpins and cod were transferred in the summer to the deep water of — 1.73°, it seems clear that the adjustments are conditioned by the change in temperature, rather than by the presence of ice. Whether or not seasonal changes take place in the fishes at the bottom, which live under perfectly constant temperature conditions is not known, but it seems improbable.

Freezing point of invertebrates

A series of freezing point determinations was made in the blood obtained from various marine invertebrates. The summer data are given in table 5. It will be seen that they

TABLE 4

Plasma freezing point of shore fishes in Hebron Fjord. Winter. Water temperature —1.73°. Depth 2–8 m

Gadus ogac	—1.36	—1.36	—1.41	—1.49	—1.51	—1.52	—1.56	—1.57
Myoxocephalus scorpius	(—1.16)	—1.36	—1.43	—1.55	(—1.60	—1.54 Baffinland)		

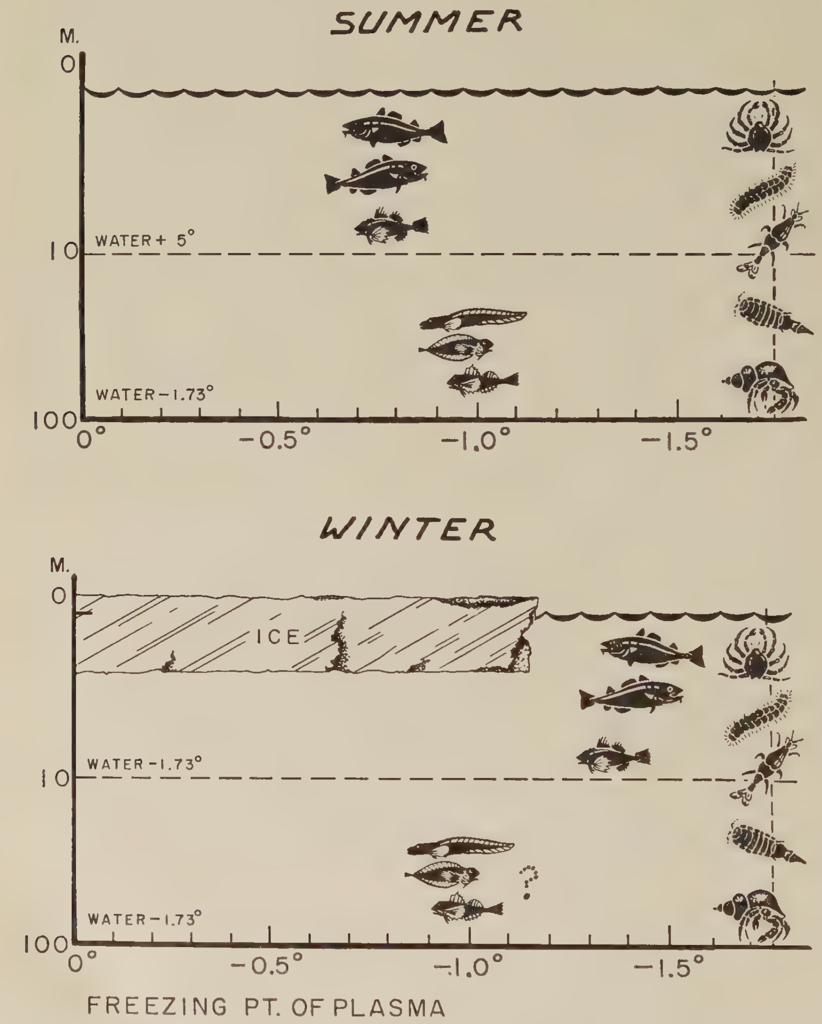

Fig. 4 Freezing point of plasma in shallow water fishes (fjord cod and sculpin) and in deep water fishes *(Lycodes, Liparis, Gymnacanthus)*. Position of fishes on abscissa indicates the freezing point of their plasma.

cluster closely around the osmoconcentration of the sea water. Two observations were made in the winter on spider crabs (*Hyas araneus*) taken at a depth of 1 meter under the ice. The freezing points were again close to that of sea water, namely, — 1.68° and — 1.73°, as would be expected. Thanks to the isosmosity of arctic as well as non-arctic marine invertebrates they face no danger of freezing except when trapped and frozen into the fjord ice, or when frozen entirely out of water. Even so, many tidal forms have been shown to stand a large amount of internal freezing (Kanwisher, '55).

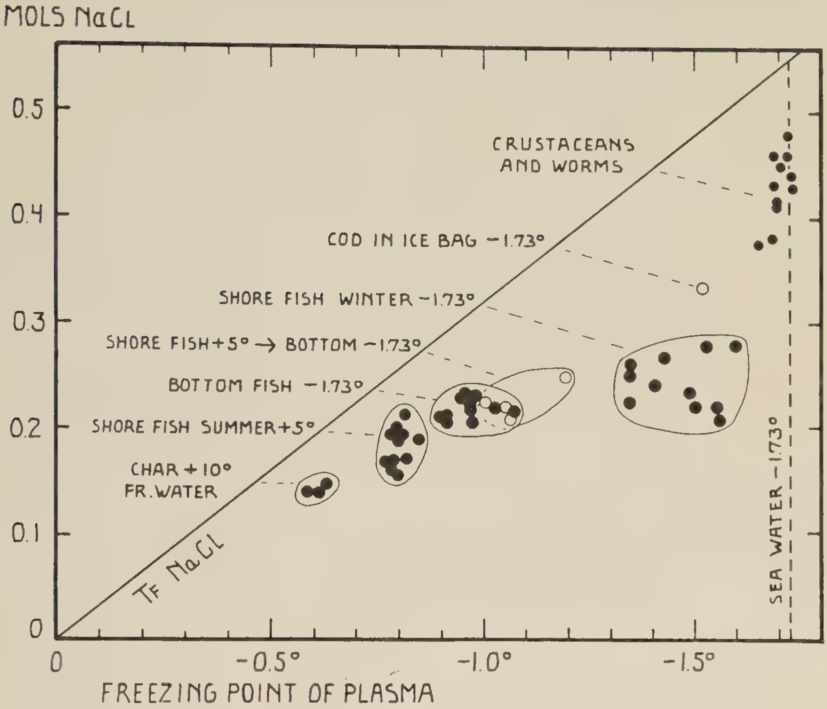

Fig. 5 Freezing point versus chlorides in fishes and invertebrates from Hebron Fjord. Shallow water fishes are here called shore fishes.

Osmoregulation by chlorides

Most of the plasma samples taken for freezing point determinations were also analyzed for chlorides (fig. 5). It will be seen that the chlorides (halides) make up for about 80% of the osmotic pressure in both deep and shallow water summer fishes; i.e., those which have what might be called a normal osmoconcentration.

In the fishes which were transferred from the warm surface water to the bottom at —1.73°, the total osmoconcentration increased, but the chlorides rose relatively less, and in the great elevation in osmoconcentration which takes place in shallow water fishes in the winter the chlorides contribute only half of the freezing point depression.

A single plasma determination on the Baffinland sculpin (T_f = —1.6°) showed a high non-protein nitrogen (90 mg %), which in a compound of one nitrogen atom per molecule (such as in trimethylamine oxide) could have accounted for another 0.1° depression. Added to 0.8° for the chlorides this would give a total freezing point depression of 0.9°, leaving 0.7° still unaccounted for.

TABLE 5

Blood freezing point of invertebrates in Hebron Fjord. Summer. Water temperature —1.73°

SPECIES	100 m			200 m	
Shrimp *(Argis dentata)*	—1.68	—1.72	—1.70	—1.42	—1.65
Hermit crab *(Pagurus kröyeri)*	—1.70			—1.74	—1.60
Spider crab *(Hyas araneus)*				—1.61	
Isopod *(Saduria sabini)*	—1.68	—1.71		—1.70	—1.75
Polychaete (scale worm)	—1.67	—1.65			

It is interesting to note that the codfish which was supercooled by 0.2° and did not freeze after three days spent with a piece of ice in a flour bag at —1.73°, elevated its chlorides very markedly (fig. 5).

As in the summer fishes, the chlorides in the invertebrates are responsible for some 80% of the freezing point depression. What the other components are we do not know.

Conclusions

In the bottom waters of Hebron Fjord lives a population of fishes that are permanently supercooled by almost 1°C. If experimentally seeded with ice at their normal habitat temperature, most of them freeze and die. Ice can, of course, never reach them at the bottom, and in the absence of seeding their state of slight supercooling is stable enough to permit them to maintain their ancestral hypotonicity. Evidently spontaneous freezing takes place too rarely to be of any consequence for their survival.

Shallow water fishes, swimming about under the ice in the winter, would very likely be seeded and frozen unless they were somehow protected. They run little risk, however, because their osmoconcentration is twice as high as in the summer, which is enough to render them almost isosmotic with the sea water and hence freeze proof.

Our supercooled bottom fishes represent, we believe, the second instance known in nature where evolution has pushed life into a region of permanent physical metastability. The other case is in plants. It is generally believed that the sap in tall trees is under tension, with pressures sometimes as low as —20 atmospheres or less. The sap is therefore in a metastable and potentially almost explosive state. A return to the stable physical state would in either of these cases be fatal, but it does not occur because of the absence of proper nuclei to trigger the catastrophe.

The large rise in osmoconcentration in the winter which takes place in the arctic shallow water fishes represents a unique osmoadaptation induced by temperature, which protects the animal from freezing.

SUMMARY

A study has been made of the osmoregulation of fishes and invertebrates living at or near the freezing point of sea water. The bottom of Hebron Fjord in northern Labrador has a water temperature of —1.7° to —1.8°C. all year round. Here

lives a large population of fishes with a plasma freezing point of —0.9° to —1.0°. They are hence supercooled by —0.7° to —0.8°. When brought to the surface and put in an ice bath at —1.7° most of these fishes freeze and die. In an ice bath of this temperature surface fishes in the summer also freeze and die. Neither deep nor shallow water fishes freeze, however, when kept at the same temperature at the bottom. In supercooled sea water of —3° several species of southern fishes survive without freezing. If the water is seeded they freeze and die almost instantly. Even in water of —1.5° they freeze and die if touched with ice. It was found that seeding would propagate through pieces of fish skin in either direction. Ice will penetrate a UF glass filter with 1–2 μ pores, but not cellophane. Absence of ice seeding is therefore the condition that makes it possible for the bottom fishes to preserve their hypotonic ancestral blood, at the cost, however, of being supercooled. The shallow water fish moving about in the ice in the winter double their osmoconcentration at that time, i.e., until it almost matches the sea water, and are thereby protected against freezing. This adaptation takes place with only a slight increase in chlorides. Marine invertebrates in the summer and winter are nearly isosmotic with the sea water, and therefore, cannot freeze as long as they remain in the water.

ACKNOWLEDGMENTS

We are very much indebted to Dr. A. C. Redfield and to Dr. R. H. Backus, of the Woods Hole Oceanographic Institution, for initiating the present study and for contributing to it their advice and support. In making preparations for our expedition we wish to acknowledge the help of Admiral Edward H. Smith, Director of the Woods Hole Oceanographic Institution, who has given us the benefit of his rich arctic experience. Success in our fishing was largely due to the knowledge of Dr. Backus and of Commander David C. Nutt, Arctic Specialist at the Dartmouth College Museum and

Master of the Blue Dolphin. Commander Nutt and the crew of the Blue Dolphin rendered us invaluable help throughout the course of our summer expedition to Hebron, in transporting personnel, provisions, and equipment, in locating and setting up the land laboratory and camp, and in providing temperature data from the fjord waters. The portable plywood laboratory was designed by Mr. K. Morrison of the Woods Hole Oceanographic Institution. It was easy to erect and served its purpose admirably. We are indebted to the U. S. Navy Bureau of Aeronautics for placing at our disposal a target-towing winch. This was skillfully adapted to a trawl winch by Mr. Warren O. Bowman and Mr. Leonard S. Perry of the Woods Hole Oceanographic Institution. Mr. Peter Hay of Nain, Labrador, ably assisted us in all phases of our summer work at Hebron. We wish to thank for their fine cooperation Major Willie Knutsen, U.S.A.F., of the Survival School at the U. S. Air Force Base, Goose Bay, Labrador, Lt. W. G. Swart, U.S.A.F., commanding officer of the U. S. Air Force Detachment at Frobisher Bay, Baffinland, and Constable R. D. Van Norman, of the Royal Canadian Mounted Police, at the same base. We are likewise much indebted to the Reverend F. C. P. Grubb, of Hebron, Labrador, for giving us every possible facility and help on our last winter expedition. For identification of the crustaceans and the isopod we wish to thank Dr. Fenner A. Chace, Jr., and Dr. Thomas E. Bowman of the Smithsonian Institution.

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KINETIC STUDIES OF PIGMENT SYNTHESIS BY NON-SULFUR PURPLE BACTERIA[1]

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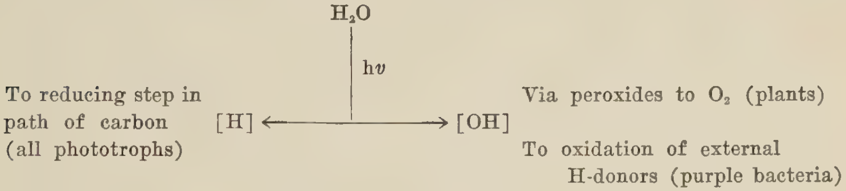
TWENTY-ONE FIGURES

The non-sulfur purple bacteria (family Athiorhodaceae) carry out a special type of photosynthetic metabolism, in which the oxidized moiety arising from the primary photochemical split of water is used to oxidize an external hydrogen donor, instead of giving rise to molecular oxygen as in green plants. The essential difference between the two kinds of photosynthesis can be schematized as follows:


H_2O
$h\nu$
To reducing step in path of carbon (all phototrophs)
[H]
[OH]
Via peroxides to O_2 (plants)
To oxidation of external H-donors (purple bacteria)

Many different external hydrogen donors can be used by nonsulfur purple bacteria; the principal ones are simple organic compounds, such as the aliphatic acids and alcohols. The absence of oxygen production makes it possible for such photosynthesis to occur under strictly anaerobic conditions, and many strains of non-sulfur purple bacteria are, in fact, obligate anaerobes. Since they do not have fermentative mechanisms of substrate decomposition which permit anaerobic growth in the dark, such strains are also obligate phototrophs. Other strains, however, grow well in the presence of air. The facultatively aerobic representatives of the family are able to fulfill the energy requirements for growth in two ways, since under aerobic conditions in the dark they can use as substrates for oxidative metabolism the same organic compounds which serve them as hydrogen donors for photosynthesis in anaerobiosis and light.

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It has long been known that the pigment content of facultatively aerobic strains varies greatly with growth conditions. In his monograph on the Athiorhodaceae, van Niel ('44) summarized existing information about these color changes as follows:

''The environmental conditions may considerably influence the composition of the pigment system of purple bacteria. The most striking effects are no doubt exerted by light and oxygen. As a rule pure cultures grown aerobically in the dark produce only little pigment . . . The more restricted the oxygen supply in such cases, the more pronounced is also the pigmentation . . . The most abundant pigmentation occurs, however, in illuminated cultures, where it seems little, if at all, influenced by oxygen . . . In view of the fact that oxygen does not participate in the metabolism of suspensions of non-sulfur purple bacteria in the light, the relative independence of pigment production from oxygen tension is not surprising, although admittedly conclusions drawn from experiments with non-growing bacteria need not hold true for cultures in which multiplication takes place . . . What has been here remarked concerning the effects of environmental conditions on pigment formation is based entirely on visual observations. No careful quantitative studies have yet been made on the pigments of the Athiorhodaceae.''

For such studies, it is essential to work under controlled conditions of growth; and, although a few quantitative investigations on the pigments of the Athiorhodaceae have been made since the appearance of van Niel's monograph (van Niel, '47; Goodwin and Osman, '53; '54; Goodwin, Land and Osman, '55), this essential requirement has in no case been fulfilled. It is our purpose to describe here the techniques of such controlled growth experiments, together with some general observations on the kinetics of pigment synthesis by two species on non-sulfur purple bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum.*

Biological material

Most of the experiments to be described were performed with the wild type of *Rhodopseudomonas spheroides,* strain 2.4.1 (obtained from the collection of Professor C. B. van Niel) and with a spontaneous green mutant derived from it, strain 2.4.1 Ga. This species is particularly well suited for kinetic work on pigment synthesis under varied environmental conditions, since it is capable of more vigorous aerobic growth than the other non-sulfur purple bacteria that we have examined.

Cultures of the wild type grown photosynthetically are brown in color. The absorption spectrum of a cell suspension is shown in figure 1. The peaks at 450, 477 and 510 mμ are caused by a mixture of two carotenoids, one yellow and one red, which were isolated and partly characterized by van Niel ('47). The peaks at 590, 805 and 855 mμ, together with the shoulder at about 875 mμ, are caused by bacteriochlorophyll. Photosynthetic cultures of the mutant strain are bright green; in the spectrum of the cells (also shown in fig. 1) the pattern of light absorption is almost identical with that of the wild type except in the carotenoid region, where there is a general shift of the peaks about 20 mμ towards shorter wavelengths. This shift reflects the replacement of the carotenoids present in the wild type by two new yellow carotenoids, which have been identified by Nakayama (unpublished observations) as neurosporene and a dihydroxy derivative of it. Apart from their differences in carotenoid content, the wild type and the green mutant resemble each other very closely. In many experiments we have used the green mutant in preference to the wild type on account of the fact that the total carotenoid content of the cells can be determined more simply and accurately.

A few experiments, mostly designed to establish the general validity of the results obtained with *R. spheroides,* were performed with a second species of non-sulfur purple bacterium, *Rhodospirillum rubrum.* Strain 1.1.1 from Professor van Niel's collection was used. The absorption spectrum of the cells (fig. 1) shows chlorophyll peaks at 590, 805 and 880 mμ, together with a broad region of light absorption by carotenoids between 450 and 550 mμ.

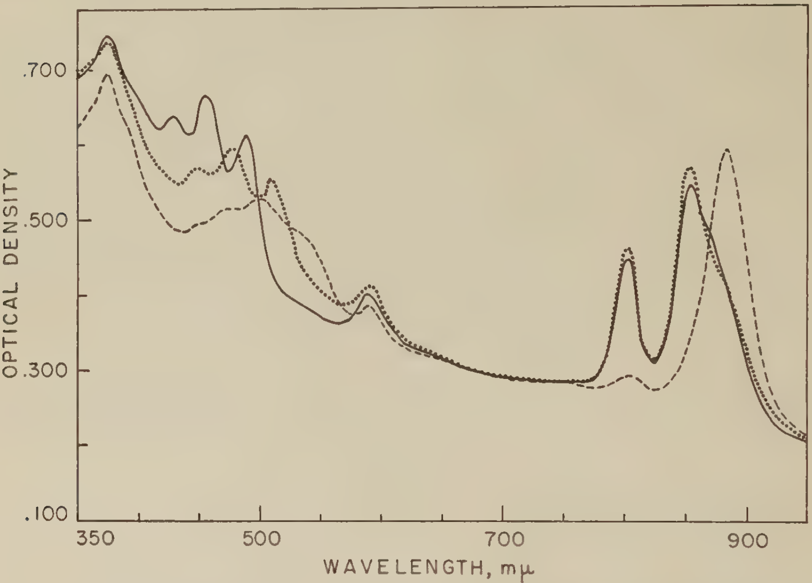

Fig. 1 Absorption spectra of whole cells of the three strains of purple bacteria employed in kinetic studies. The cells were taken from cultures in the exponential phase of growth under anaerobic conditions at the same light intensity. All three spectra have been adjusted to the same optical density at 680 mμ. . . . . *Rhodopseudomonas spheroides,* wild type; ——— *Rhodopseudomonas spheroides,* green mutant; – – – – – *Rhodospirillum rubrum.*

Culture media

Stock cultures were maintained as stabs incubated in the light on a solid medium of the following composition: Difco yeast extract, 0.3 gm; Difco casamino acids, 0.2 gm; agar, 1.5 gm; and tap water, 100 ml.

In the growth experiments, a semi-synthetic medium based on that developed some years ago by Hutner ('50) was employed. This medium has been modified subsequently by Hutner (personal communication) and further slightly changed by us; the current version used in our laboratory will be given. Most of the ingredients are made up beforehand, in the form of the following three stock solutions:

1.	Potassium phosphate, pH 6.8	1.0 *M*
2.	Ammonium DL-malate, pH 6.8	1.0 *M*
3.	Concentrated base:	
	Nitrilotriacetic acid	10.0 gm
	$MgSO_4$	14.45 gm
	$CaCl_2.2H_2O$	3.335 gm
	$(NH_4)_6Mo_7O_{24}.4H_2O$	9.25 mg
	$FeSO_4.7H_2O$	99.0 mg
	Nicotinic acid	50.0 mg
	Thiamin·HCl	25.0 mg
	Biotin	0.5 mg
	Metals ''44''	50.0 ml
	Distilled water to 1000 ml.	

In preparing solution 3, the nitrilotriacetic acid is dissolved and neutralized with KOH (about 7.3 gm), after which the rest of the ingredients are added and the pH is adjusted to 6.6–6.8 before making to volume. Metals ''44'' contains per 100 ml:

Ethylenediaminetetraacetic acid	250.0 mg	
$ZnSO_4.7H_2O$	1095.0 mg	(250 mg Zn)
$FeSO_4.7H_2O$	500.0 mg	(100 mg Fe)
$MnSO_4.H_2O$	154.0 mg	(50 mg Mn)
$CuSO_4.5H_2O$	39.2 mg	(10 mg Cu)
$Co(NO_3)_2.6H_2O$	24.8 mg	(5 mg Co)
$Na_2B_4O_7 10H_2O$	17.7 mg	(2 mg B)

A few drops of sulfuric acid are added to retard precipitation.

In order to prepare one liter of the complete medium, 20 ml each of solutions 1, 2 and 3 are mixed and diluted with distilled water, after which 1.0 gm of vitamin-free casamino acids (Difco) is added. A copious precipitate forms when this medium is autoclaved but redissolves on cooling. The casamino acids may be replaced by a mixture of L-glutamic acid (0.1% final concentration) and sodium acetate $.3\,H_2O$ (0.1% final concentration). The medium is perfectly clear and colorless, and supports growth at a rate equal to that in a yeast extract medium.

Growth conditions

Inocula for kinetic experiments were obtained from cultures in the exponential phase of growth on the semi-synthetic medium described in the preceding section. Such cultures were grown either anaerobically in the light or aerobically in the dark. The light-grown cells were cultivated in 50 ml Florence flasks, filled to the neck and incubated in a light cabinet. The dark-grown cells were cultivated in 250 ml Erlenmeyer flasks containing 30 ml of medium, and agitated mechanically to provide the necessary aeration. The temperature of incubation was 30°C. in both cases.

For the growth experiments proper, the concentration of ammonium malate was lowered to 0.01 *M,* in order to ensure that growth would eventually be limited by the amount of hydrogen donor available. For the most part, especially designed culture vessels of rectangular shape were employed. They measure 15 × 10 × 3 cm, and have a cylindrical neck 6 cm in length into which can be inserted a rubber stopper equipped with gas inlet, gas outlet and sampling tubes. The stopper assembly is separately sterilized, wrapped in brown paper. In some experiments large test-tubes (2.8 × 20 cm), fitted with rubber stoppers similarly equipped, were used in place of the rectangular growth flasks. The sterilized culture vessels were filled with medium, previously inoculated at the desired optical density with cells in the exponential phase of growth, and placed in a rectangular glass water bath which was maintained at a temperature of 30° C. Continuous aeration was conducted either with 95% N_2-5% CO_2 (anaerobic experiments) or 95% air-5% CO_2 (aerobic experiments). Illumination was provided by one or two 300W flood lamps, adjusted so as to give a uniform light intensity on the entrance window of the bath, and therefore also on the surface of the culture flasks, which were placed at a fixed distance (0.5 cm) from the entrance window in most experiments. Light intensity was adjusted by changing the distance of the lamps from the bath. A Weston light meter was used to determine light intensities.

Growth measurement

The increase in cell mass was estimated by measuring the optical density at 680 mμ of bacterial suspensions in 1 cm cuvettes, using a Beckman DU spectrophotometer. The chosen wavelength lies between the main regions of light absorption by the photosynthetic pigments (fig. 1), and, as shown by comparisons with total and viable counts, the $\text{O.D.}_{680\ \text{m}\mu}$ furnishes an accurate measure of cell mass with either pigmented or unpigmented cells. The relationship between concentration and optical density is linear up to an optical density of 0.300. Cell suspensions of higher optical densities were diluted with water or 0.02 M phosphate buffer before being read. An optical density of 1.000 corresponds to a concentration of about 4×10^9 bacteria per milliliter, or to a dry weight of 1.134 mg per millimeter, in the case of *R. spheroides.*

Determination of growth yields

Figure 2 shows the growth yields obtained under conditions of photosynthetic and aerobic development with the green mutant of *R. spheroides* in the semi-synthetic medium furnished with different concentrations of malate as the principal carbon source, together with a fixed concentration of vitamin-free casein hydrolysate (0.02%). The aerobic cultures were grown in 40 ml of medium in 500 ml Erlenmeyer flasks, shaken mechanically on a rotary shaker at 30°C. The photosynthetic cultures were grown in completely filled Florence flasks, incubated in a light cabinet at 30°C. Under these conditions, maximal populations were reached in the aerobic cultures after 48 hr. and in the photosynthetic ones after 72 hr. It can be seen that the growth yield is proportional to malate concentration up to a value of 0.1 M. At any given malate concentration, the cell mass obtained in a photosynthetic culture is nearly twice that obtained in an aerobic one. Determinations of dry weight gave the following results: 2.1 mg of malic acid yield 1 mg of bacterial cells under photosynthetic conditions, whereas 3.77 mg of malic acid are required to yield the same dry weight of bacteria under aerobic conditions. Assuming that the carbon content of the dry cells is 50%, it follows that during photosynthetic growth 68% of the malic acid carbon is assimilated into cell material, whereas during aerobic growth only 37% is assimilated. These results are in very good agreement with Clayton's ('55) estimations of carbon assimilation from malic acid by resting

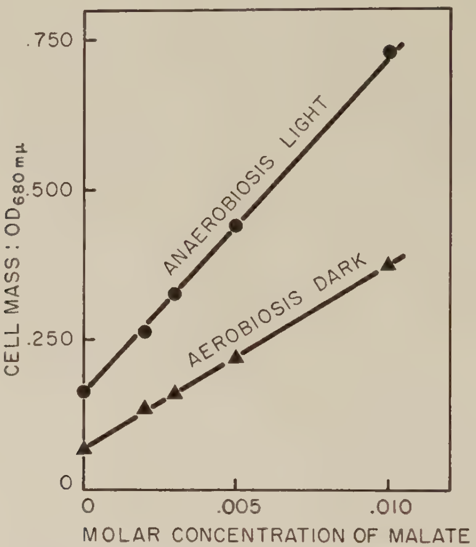

Fig. 2 Photosynthetic and respiratory growth yields of the green mutant of *Rhodopseudomonas spheroides* as a function of malate concentration in the semi-synthetic medium used for growth experiments. The medium contained 0.02% of vitamin-free casein hydrolysate, which is capable of supplying small amounts of organic hydrogen donors to the cell, and hence the growth yields do not fall to zero at zero concentration of malate.

cells of *R. rubrum.* He found a carbon assimilation of 37% during respiration, and of 69% during photosynthesis.

Chlorophyll estimation in intact cells

As shown in figure 1, the bacteriochlorophyll in the cells of *R. spheroides* shows absorption maxima at 805, 855 and about 875 mμ. The magnitudes of these three chlorophyll maxima can be roughly estimated as follows. A scattering curve is determined upon a suspension of unpigmented cells (grown under strictly aerobic conditions). Since pigmented and unpigmented cells have the same optical density at 680 mμ, the ratio of the optical density at 680 mμ to the optical densities at the three wavelengths corresponding to the chlorophyll maxima, as determined upon the unpigmented cells, can be applied to suspensions of pigmented cells in order to correct for light scattering the optical densities observed at the chlorophyll maxima. This method is useful for following changes in the relative intensities of the three chlorophyll peaks during growth, or for making quick and simple estimations of chlorophyll synthesis. It cannot be successfully extended to measurement of light absorption by carotenoid pigments *in vivo,* since the scattering is too great in the blue end of the spectrum, where their bands are located.

Extraction and determination of bacteriochlorophyll and carotenoids

Methods were developed for the quantitative determination of bacteriochlorophyll and carotenoids in bacterial suspensions containing a total of about 10^{10} cells, and these methods were applied to the measurement of pigment synthesis during growth. An accurately measured sample of the culture was centrifuged and the supernatant liquid discarded. After the centrifuge tube had been carefully drained, the packed cells were resuspended in a small volume of water, and the pigments then extracted by the addition of an appropriate solvent. The suspension was again centrifuged to sediment the extracted cells, and the supernatant was used as soon as possible for the spectrophotometric determination of the pigments.

In experiments with the green mutant of *R. spheroides*, the packed cells were resuspended in 0.25 ml of water, and the pigments extracted by the addition of 4.75 ml of absolute methanol. The same procedure could not be used for the wild type of *R. spheroides* and for *R. rubrum*, since their carotenoids are not completely extracted by aqueous methanol. Accordingly, in experiments with these two organisms, the packed cells were resuspended in 0.1 ml of water, and the pigments extracted by the addition of 4.9 ml of a mixture consisting of 7 parts of acetone and 2 parts of methanol (v/v). The determination of the pigments was made as soon as possible after extraction, since bacteriochlorophyll is relatively unstable in organic solvents, particularly if exposed to light.

The following calculations have been made for an initial sample of the culture with a volume of 5 ml, and must accordingly be multiplied by a factor if a different volume is taken.

Procedure with extracts of the green mutant of R. spheroides

The optical density of the methanolic extract is measured at 775 mμ and at 468 mμ. In methanol, the extinction for pure bacteriochlorophyll at 775 mμ ($E^{1\%}_{1\,\mathrm{cm}}$) is 0.387×10^3. Since there is no carotenoid absorption in this region, the amount of chlorophyll in milligrams per 100 ml of culture can then be calculated directly: it is equal to $\mathrm{O.D.}_{775} \times \frac{1}{E^{1\%}_{1\,\mathrm{cm}}} \times 10^3$, or $\mathrm{O.D.}_{775} \times 2.58$. The carotenoids of the green mutant, neurosporene and its dihydroxy derivative, have identical spectra and almost identical extinction coefficients: at 468 mμ in methanol, $E^{1\%}_{1\,\mathrm{cm}}$ is 2420 for neurosporene and 2220 for dihydroxyneurosporene. Since the cells contain almost equal amounts of these two pigments, one can use the average value of 2320 for purposes of calculation. However, bacteriochlorophyll also absorbs to a certain extent at 468 mμ, and the observed optical density must be corrected for the amount of light absorbed by bacteriochlorophyll before calculation of the carotenoid concentration; the correction is made by subtracting 0.1275 × $\mathrm{O.D.}_{775}$ from the observed $\mathrm{O.D.}_{468}$. The total amount of both carotenoids in milligrams per 100 ml of culture is then obtained from the formula:

corrected $\mathrm{O.D.}_{468} \times \frac{1}{E^{1\%}_{1\,\mathrm{cm}}} \times 10^3$, or corrected $\mathrm{O.D.}_{468} \times 0.431$.

Procedure with extracts of the wild type of R. spheroides and R. rubrum

The extinction of pure bacteriochlorophyll ($E^{1\%}_{\mathrm{cm}}$) at 775 mμ in acetone-methanol is 0.457×10^3. Therefore, the chlorophyll content in milligrams per 100 ml of culture is obtained from the expression $\mathrm{O.D.}_{775} \times \frac{1}{E^{1\%}_{1\,\mathrm{cm}}} \times 10^3$, or $\mathrm{O.D.}_{775} \times 2.19$. The wild type of *R. spheroides* contains two major carotenoids which were crystallized by van Niel ('47), who determined their extinctions in benzene. In order to determine their extinctions in acetone-methanol, we separated the two pigments chromatographically, using the procedures of van Niel ('47), and determined the light absorption by a sample of each pigment both in benzene and in acetone-methanol. There is considerable overlapping between their spectra. Consequently it is necessary to measure absorption at two wavelengths (456 and 510 mμ), and, after subtracting light absorption at these wavelengths caused by bacteriochlorophyll, to apply simultaneous equations to the corrected optical densities in order to determine the amount of each carotenoid. The chlorophyll correction at 456 mμ is $\mathrm{O.D.}_{775} \times 0.1$, and at 510 mμ is $\mathrm{O.D.}_{775} \times 0.05$. After making these corrections, the quantities of the yellow and red carotenoid in milligrams per 100 ml of culture can be obtained by the following simplified calculations:

Yellow carotenoid: (corrected $\mathrm{O.D.}_{456} \times 0.397$) — (corrected $\mathrm{O.D.}_{510} \times 0.355$).

Red carotenoid: (corrected $\mathrm{O.D.}_{510} \times 0.692$) — (corrected $\mathrm{O.D.}_{456} \times 0.0728$).

Although in old cultures of *R. rubrum* spirilloxanthin is the principal carotenoid present in the cells, this is not true during the early stages of growth (Goodwin and Sissins, '55). In view of the qualitative changes of carotenoid constitution which occur in this organism, we were unable to develop a satisfactory method for carotenoid measurement during growth, and hence quantitative data on bacteriochlorophyll alone are given for *R. rubrum.*

Effect of light on pigment synthesis

We shall describe first the general effects of light on photosynthetic growth and the manufacture of bacteriochlorophyll and carotenoids by the cell. Two principal types of response have been studied: the response of cells growing under photosynthetic conditions, and hence already endowed with a relatively high content of photosynthetic pigments — so-called "pigmented cells" — to sudden changes of light intensity; and the response of cells grown aerobically in the dark, and consequently possessing a very low pigment content — so-called "unpigmented cells" — to enforced photosynthetic development at different light intensities.

The essential features of both kinds of response are illustrated by an early experiment, which is very instructive, although technically crude. Four tubes were inoculated at the same initial optical density with cells of the wild type of *R. spheroides* in the phase of exponential growth. The inoculum for cultures A and C consisted of pigmented cells, grown anaerobically in the light, whereas the inoculum for cultures B and D consisted of unpigmented cells, grown aerobically in the dark. All 4 cultures were incubated in the light and aerated with 95% N_2 — 5% CO_2. A and B were placed close to the entrance window of the water bath and consequently received relatively strong illumination (*ca.* 600 foot-candles). C and D were placed at the far end of the water bath from the entrance window, and hence received much less light (less than 200 f.-c.s.). Growth was measured by following the change of optical density at 680 mμ, and chlorophyll synthesis was roughly estimated by measuring the *in vivo* light absorption at the main infra-red peak (855 mμ), corrected for scattering as described earlier.

As shown in figure 3, growth in A and C, inoculated with pigmented cells, was immediate. The initial growth rate in C, exposed to a dim light, was considerably less than in A,

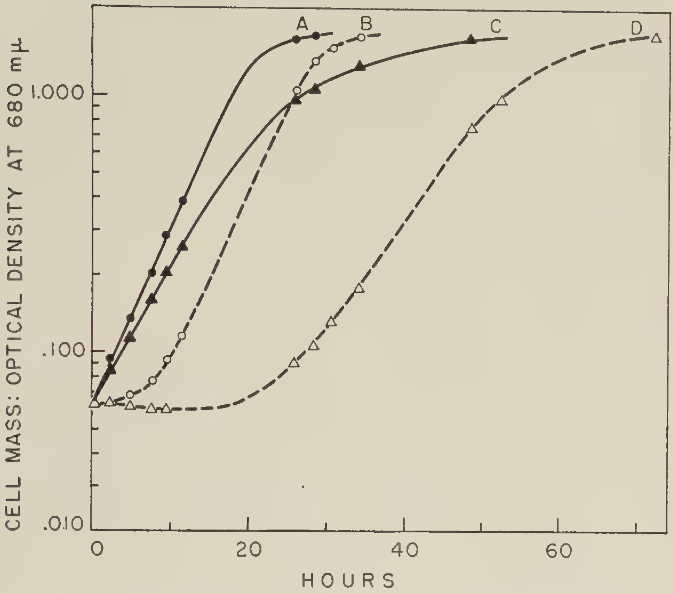

Fig. 3 Growth of the wild type of *Rhodopseudomonas spheroides* under anaerobic conditions at two different light intensities. Cultures A and B were grown in bright light, cultures C and D in dim light. Cultures A and C were inoculated with cells in the exponential phase of photosynthetic growth, cultures B and D with cells in the exponential phase of aerobic growth in the dark.

and as the overshadowing of the cells in culture C increased with growth, its growth rate diminished. Cultures B and D, inoculated with unpigmented cells, started to grow only after a lag, whose duration was inversely related to light intensity. Eventually, all 4 cultures entered the stationary phase at the same level of optical density, since the total growth was limited by the amount of malate furnished. The patterns of growth observed are in accordance with expectation: cells adapted previously to photosynthetic growth continued to grow without lag, at rates governed by the light intensity, whereas cells previously grown in the dark, and hence equipped with a weak complement of photosynthetic pigments, had to undergo a period of adaptation before they could begin photosynthetic growth. At the end of the experiment, cultures C and D, grown in dim light, were considerably more pigmented than cultures A and B, grown in bright light. Figure 4 shows the kinetics of chlorophyll synthesis in all 4 cultures. In this graph, the amount of chlorophyll (roughly estimated from light absorption at 855 mμ) is plotted as a function of the cell mass, and the slopes of the curves therefore represent differential rates of chlorophyll synthesis. In culture A, inoculated with pigmented cells and placed in bright light, no chlorophyll was synthesized for more than one generation;

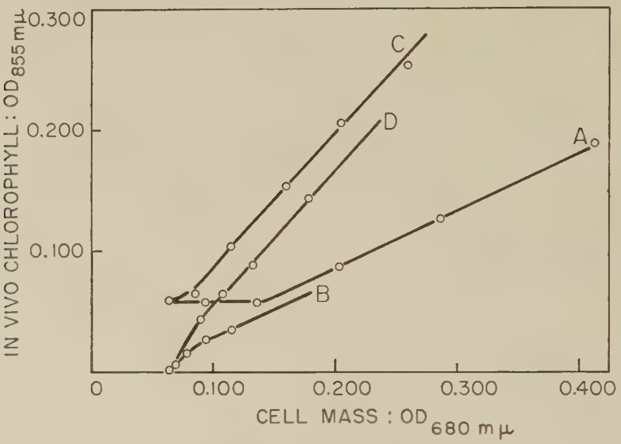

Fig. 4 The synthesis of chlorophyll as a function of the increase of cell mass in the four cultures for which growth curves are shown in figure 3. Chlorophyll was estimated *in vivo* by optical density measurements at 855 mμ, corrected for scattering.

A:	Bright light	Inoculum grown anaerobically in the light
C:	Dim light	
B:	Bright light	Inoculum grown aerobically in the dark
D:	Dim light	

thereafter, synthesis resumed at a constant differential rate (0.38 in arbitrary units). In culture C, started from an inoculum identical with that of culture A but placed in much dimmer light, pigment synthesis began almost coincidentally with growth, and proceeded at a much higher steady differential rate (1.07 in arbitrary units). In cultures B and D, inoculated with unpigmented cells, there was an extremely high initial differential rate of chlorophyll synthesis, which subsequently fell to a steady value, characteristic for each culture. In culture B, the steady value was identical with that in culture A, while in culture D, it was identical with that in culture C; thus, irrespective of the initial pigment content and the behavior during the primary phase of adjustment, the same steady state rate of chlorophyll synthesis is eventually established at any given light intensity. The most striking aspect of pigment synthesis is its first phase, during which the cells adjust their pigment content in response to the light intensity which they are receiving. For cells which have a higher initial pigment content than that required by the light intensity, adjustment is brought about by a temporary suppression of pigment synthesis during growth (culture A); for cells with a lower pigment content than that required, adjustment is brought about by a temporary very rapid synthesis (cultures B and D).

Adjustment of pigment content to light intensity by photosynthetically grown cells

The following experiment, performed with the green mutant of *R. spheroides,* shows with more detail and precision the adjustment of pigment content to light intensity by photosynthetically grown cells. Two populations of cells, previously growing in dim and bright light, respectively, served as the inocula for two cultures which were allowed to develop photosynthetically at a common, intermediate, light intensity. Figure 5 A shows the response in terms of growth, chlorophyll and carotenoid synthesis which occurred with the cells exposed to an increased light intensity. Growth proceeded exponentially from the start, but chlorophyll synthesis was completely inhibited for 2.5 hours, and carotenoid synthesis for 1 hour. Thereafter, the rates of synthesis of both pigments gradually increased until they reached a rate identical with the growth rate. Figure 5 B shows the reverse situation, i.e., the response of cells exposed to a lowered light intensity. Such cells do not at the start contain enough pigment to grow photosyn-

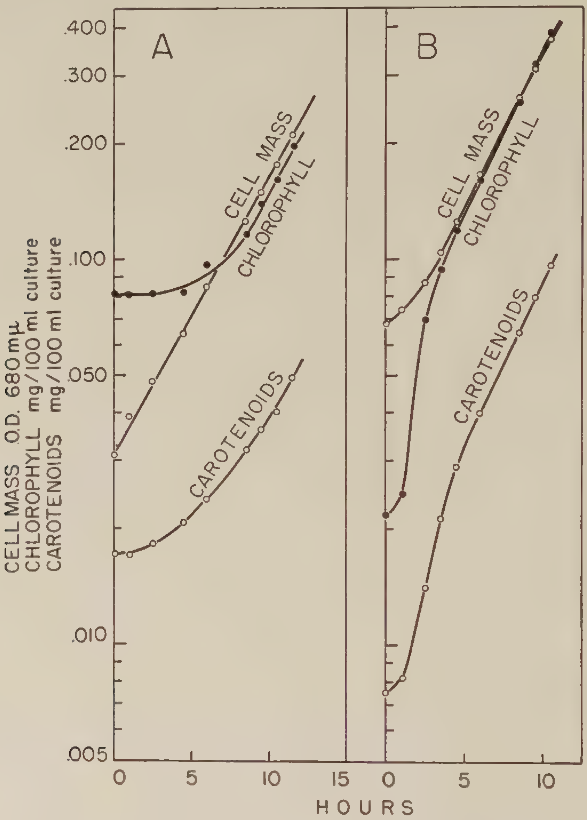

Fig. 5 The effect of sudden changes of light intensity, under anaerobic conditions, on growth, chlorophyll and carotenoid synthesis by the green mutant of *Rhodopseudomonas spheroides.* A: cells previously growing exponentially in very dim light (50 f.-c.s), and exposed at zero time to moderate light intensity (540 f.-c.s). B: cells previously growing exponentially in very bright light (ca. 5000 f.-c.s), and exposed at zero time to moderate light intensity (540 f.-c.s).

thetically at the maximal rate corresponding to this light intensity; growth therefore shows a slight lag, during which chlorophyll and carotenoid synthesis occur very rapidly. As the pigment concentration rises, the growth rate increases; eventually, the rates of growth and pigment synthesis become parallel. During the initial phase of adjustment, carotenoid synthesis is slightly less rapid than chlorophyll synthesis.

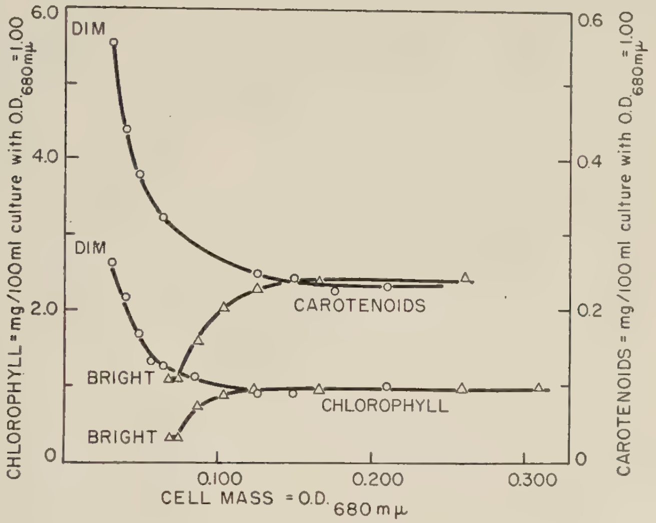

Fig. 6 The regulation of the photosynthetic pigment content of the cell by light intensity under anaerobic conditions. The data from the growth experiments with the green mutant of *R. spheroides* illustrated in figure 5 have been replotted to show how the chlorophyll and carotenoid concentrations in the cell change as a function of the increase in cell mass. The curves marked DIM show the changes of pigment concentration in the culture exposed to a sudden increase of light intensity: those marked BRIGHT show similar changes in the culture exposed to a sudden decrease of light intensity. Both cultures grew at the same light intensity, and consequently entered steady state conditions of pigment synthesis (as indicated by growth unaccompanied by further changes of pigment concentration) at identical pigment concentrations.

In figure 6 the data from these two experiments have been combined and replotted to show the changes in pigment concentration per unit cell mass which occur during adjustment to a common light intensity. It is evident that the intracellular concentrations of the photosynthetic pigments reach the same values in both cultures after the phase of adjustment, which involves either dilution or preferential synthesis, depending on the previous culture history. *Rhodospirillum rubrum* shows the same type of response to a sudden change of light intensity with respect to chlorophyll synthesis, but we have no quantitative data on carotenoid synthesis for this species.

In addition to its general regulatory rôle, light intensity affects the pigment system of *R. spheroides* in two further ways. Firstly, it modifies the chlorophyll/carotenoid ratio. A change of light intensity does not affect the synthesis of the two classes of pigments in precisely the same way, although there is a broad parallelism. For example, when light intensity is abruptly increased, the suppression of carotenoid synthesis lasts for a shorter period than the suppression of chlorophyll synthesis (fig. 5 A); as a consequence the chlorophyll/carotenoid ratio under steady state conditions of pigment synthesis declines with increasing light intensity (table 1). The second effect observed with *R. spheroides* is a modification of the infra-red absorption spectrum of the cells. There are three peaks in this region of the spectrum, at 805, 855 and 875 mμ. After growth at high light intensities, the peak at 875 mμ becomes very prominent, whereas in cells grown at low light intensities it is barely detectable, being represented

TABLE 1

Chlorophyll:carotenoid ratios in the green mutant of R. spheroides after growth at different light intensities

LIGHT INTENSITY, FOOT-CANDLES	PIGMENT CONCENTRATION, MG/100 ML OF A CULTURE WITH O.D.$_{680}$ OF 1.000		RATIO, $\frac{\text{CHLOROPHYLL}}{\text{CAROTENOIDS}}$	
	Chlorophyll	Carotenoids	Weight	Molar
50	2.65	0.555	4.8	2.9
540	1.00	0.24	4.2	2.5
5000	0.32	0.11	3.0	1.8

Molar ratios were calculated assuming a molecular weight of 910 for bacteriochlorophyll, and an average molecular weight of 552 for neurosporene and dihydroxyneurosporene.

only by a slight asymmetry on the shoulder of the peak at 855 mμ. The magnitude of this change is shown in figure 7, a plot of the spectra of whole cells of the wild type *R. spheroides* measured at the beginning and end of a kinetic experiment on the effect of increasing light intensity. Both spectra have been adjusted to the same optical density at 680 mμ.

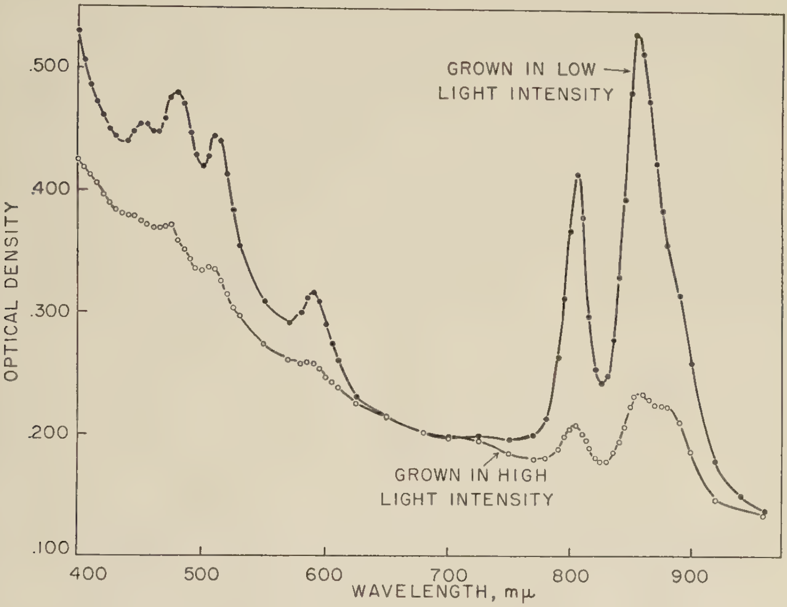

Fig. 7 Absorption spectra of whole cells of the wild type of *Rhodopseudomonas speroides* after growth in dim and in bright light. Both spectra adjusted to the same optical density at 680 mμ.

The cells grown in dim light, which served as the inoculum, are separated from the bright light cells by less than three generations of growth.

Similar modifications of the infra-red spectrum of the cells after growth at different light intensities have been observed in a purple sulfur bacterium, *Chromatium sp.,* by Wassink, Katz and Dorrestein ('39). Like *R. spheroides,* this organism has a multiplicity of infra-red peaks which cannot be correlated with peaks in the *in vitro* spectrum of bacteriocholorophyll;

Wassink, Katz and Dorrestein suggested that they might correspond to different chlorophyll-protein conjugates in the cell. If this interpretation is correct, it follows that the shifts in relative magnitude of the infra-red peaks with changing light intensity reflect light-induced changes in the differential rates of synthesis of the several chlorophyll-proteins. It has been proposed by Calvin ('55), however, that the complex infra-red spectrum of *R. spheroides* could be caused by electronic coupling of chlorophyll and carotenoids; and on this hypothesis, the changes of infra-red spectrum might be simply a secondary consequence of the change in the chlorophyll/carotenoid ratio that is brought about by shifts of light intensity. In *R. rubrum,* the infra-red spectrum of the cells bears a much closer structural correspondence to the absorption spectrum of pure bacteriochlorophyll at long wavelengths, and is unchanged in shape by changes of light intensity.

Adaptation of aerobic cells to photosynthetic growth

The behavior of aerobically-grown cells when placed under conditions of obligatory photosynthesis has already been indicated in a preceding section. Figures 8 A and 8 B show the results of a more detailed experiment, performed with the green mutant of *R. spheroides.* Identical inocula from a culture growing exponentially under aerobic conditions in the dark were placed in culture flasks incubated anaerobically at two different light intensities. In bright light (fig. 8 A), synthesis of chlorophyll and carotenoids began immediately, but growth was initiated only after a lag of 6–7 hours. In dim light (fig. 8 B) the same cells showed a greatly increased growth lag (about 12 hours), and the optical density at 680 mμ actually fell slightly at first, probably owing to a decrease in cell size. There was also some lag in pigment synthesis, although it was evidently much shorter than the growth lag. During the growth of the culture in dim light, steady state conditions of pigment synthesis were never achieved, as indicated by the lack of parallelism between the growth curve and the curves for pigment synthesis. This may be attributed to the onset of overshadowing before the population had achieved the pigment content characteristic for the light intensity in question. The data in figures 8 A and 8 B have been replotted in figure 9 to show the increase in pigment as a function of the increase in cell mass. At the beginning of the experiment, differential rates of both chlorophyll and carotenoid synthesis were in both cultures practically infinite; then, as growth resumed, these rates fell to steady values, governed for each culture by the light intensity.

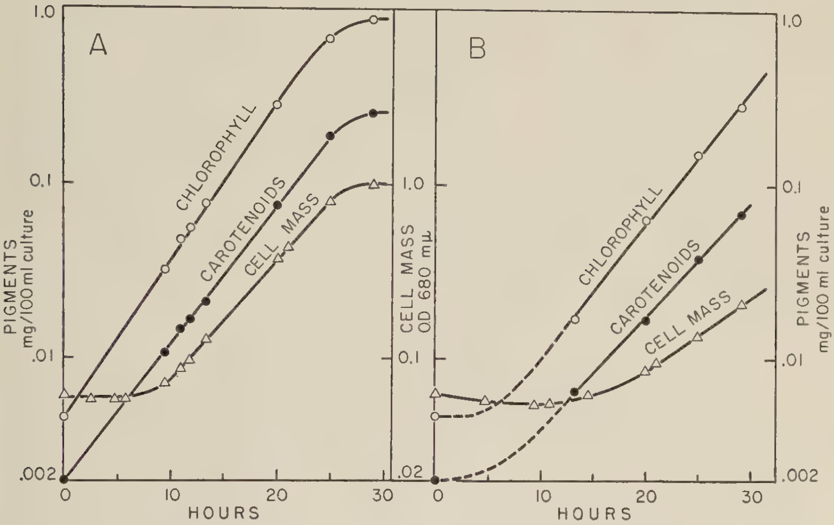

Fig. 8 Growth, chlorophyll and carotenoid synthesis by aerobically grown cells of the green mutant of *Rhodopseudomonas spheroides* when placed under anaerobic conditions at two different light intensities. A: Bright light. B: Dim light.

This experiment reveals some interesting facts about the readaptation of cells which have been depigmented by aerobic growth. The cells used as an inoculum contained small, but still detectable quantities of photosynthetic pigments. The only source of energy available to them under the environmental conditions used was that furnished by the photosynthetic function of these residual pigments. Figure 9 shows that this small amount of energy was at first channelled into the manufacture of more pigment, the general synthesis of cell material (as measured by increase of cell mass) being deferred until the restoration of the photosynthetic pigment system was well under way. It should be stressed that genetic selection plays no appreciable rôle in the adaptive process:

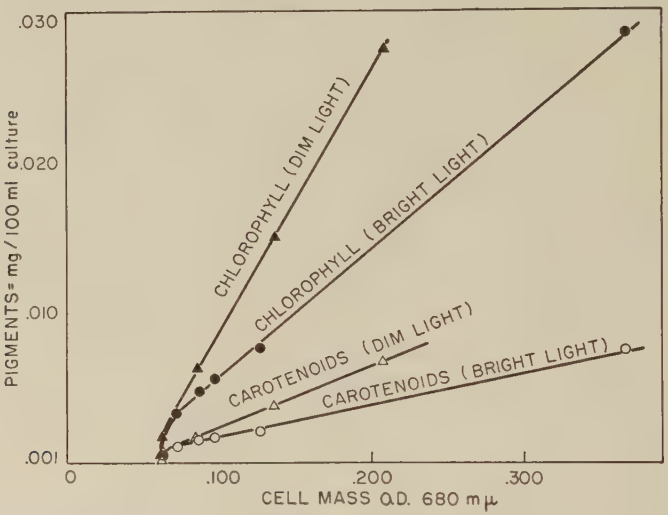

Fig. 9 Differential rates of chlorophyll and carotenoid synthesis by aerobically grown cells of the green mutant of *Rhodopseudomonas spheroides* under anaerobic conditions at two different light intensities. Data from the growth experiment illustrated in figure 8.

every cell in the population is potentially capable of synthesizing chlorophyll and carotenoids, as shown by the uniform pigmentation of colonies which develop on plates streaked from the inoculum. The duration of the growth lag in such experiments is governed by two factors: light intensity (as illustrated in fig. 8); and the initial pigment content of the cells. If the pigment content of the cells is reduced to an extremely low level by serial subculture in the dark under conditions of maximal aeration, the growth lag which follows transfer to anaerobiosis and light may last for several days.

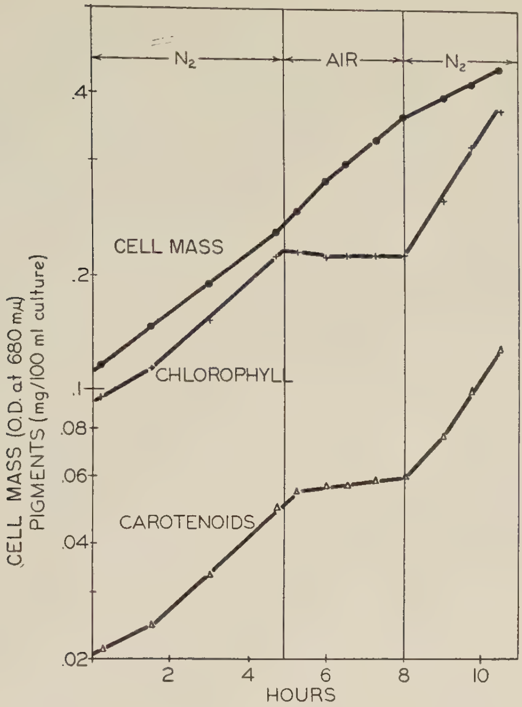

Fig. 10 The effect of air on growth, chlorophyll and carotenoid synthesis by the wild type of *Rhodopseudomonas spheroides*. Continuous illumination.

The effect of air on pigment synthesis

The growth of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* in the presence of air produces a marked bleaching of the culture, and if several successive transfers are made under conditions of really rigorous aerobiosis, the chlorophyll and carotenoids eventually diminish to barely detectable levels. We wish now to analyze the mechanism of this aerobic bleaching. The results of a typical experiment with the wild type of *R. spheroides* are shown in figure 10. Growth was initiated anaerobically in the light, and these conditions were maintained until steady state conditions of pigment synthesis had been established. At this point, the anaerobic aeration mixture (95% N_2-5% CO_2) was replaced by 95% air-5% CO_2, illumination being maintained at the original intensity. The growth rate increased slightly, no doubt owing to the simultaneous occurrence of photosynthesis and respiration. There was an abrupt and total cessation of chlorophyll synthesis, and the chlorophyll content of the growing population thereafter remained constant, within the limits of experimental error. Carotenoid synthesis diminished very markedly in rate, but did not absolutely cease. After several hours of exposure to air, the culture was returned to anaerobic conditions. The population did not go into lag, since the cells still contained enough photosynthetic pigments to grow photosynthetically, but the growth rate fell to a level well below that during the original period of anaerobic cultivation. The restoration of anaerobiosis provoked an immediate and rapid resynthesis of both photosynthetic pigments; steady state conditions would have been restored within a few hours, but the experiment was terminated too early to show this. It should be noted that in this experiment the cells were illuminated continuously throughout the period of aerobic exposure. In fact, light does not modify materially the effects of air on pigment synthesis, as illustrated by the following experiment, performed with *R. rubrum* (fig. 11). Two cultures were inoculated with photosynthetically grown cells and allowed to develop in the presence of air, one being illuminated and the other kept in the dark. The initial growth rate of the illuminated culture was slightly greater than that of the dark one, doubtless owing to the utilization of the preformed pigments for photosynthesis; but the growth rates became identical after three hours, and remained so throughout the rest of the experiment. Chlorophyll synthesis was totally suppressed in both cultures: after aerobic growth for 9.5 hours the two cultures had virtually identical chlorophyll contents, not significantly different from the chlorophyll content of the inoculum.

When a culture of *R. spheroides* is returned to anaerobiosis after a few hours of exposure to air, the resynthesis of chlorophyll starts without a detectable time lag, as illustrated in figure 10. *R. rubrum* responds in a slightly different fashion:

there is an absolute lag of approximately an hour before the onset of resynthesis. This is illustrated in figure 12, which shows the results of an experiment with *R. rubrum* exactly analogous to the experiment with *R. spheroides* portrayed in figure 10.

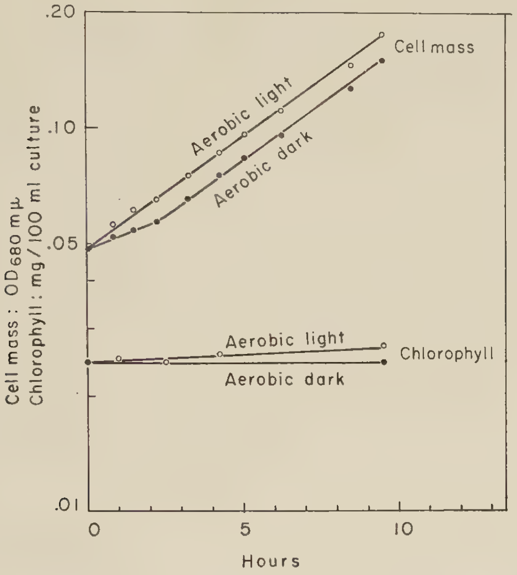

Fig. 11 The effect of air in the presence and absence of light on growth and chlorophyll synthesis by *Rhodospirillum rubrum*. Inoculum consisted of photosynthetically grown cells.

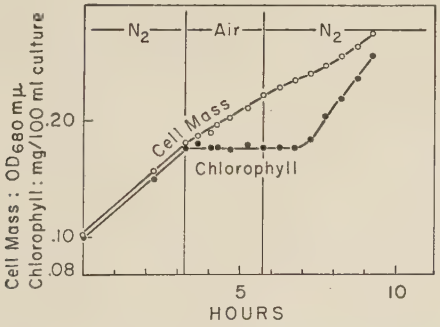

Fig. 12 The effect of air on growth and chlorophyll synthesis by *Rhodospirillum rubrum*. Continuous illumination.

Several conclusions may be drawn from the experiments presented above. In the first place, it is evident that the photosynthetic pigments of the cell are chemically stable in the presence of air, since no significant decrease in the total pigment content of the population can be detected after many hours of exposure to air, whether in the presence or absence of light. The bleaching of aerobic populations occurs exclusively by the arrest of pigment synthesis, and the consequent dilution of the initial pigment content during growth. Such populations thus eventually become physiologically incapable of photosynthesis, even if maintained at all times in the light. The powerlessness of light to halt bleaching is somewhat unexpected, since van Niel ('41) showed many years ago that light causes an almost total suppression of aerobic respiration in resting populations of *R. rubrum* grown under photosynthetic conditions. This finding has been confirmed recently by Clayton ('55). Clayton also discovered that in *R. spheroides* the effect of light on respiration is far less marked, the respiratory rate of resting cells being reduced by some 35% upon illumination. It can therefore be assumed that when air is introduced into cultures of the two species growing photosynthetically, there is a difference in the immediate metabolic effects: *R. rubrum* at first continues to carry out a well-nigh exclusively photosynthetic metabolism, whereas *R. spheroides* at once shifts to a mixed photosynthetic and respiratory metabolism. However, since chlorophyll synthesis is totally arrested, the continuation of growth inexorably entails for both organisms the eventual total replacement of photosynthesis by respiration.

The effects of air on pigment synthesis which we have just described are in contradiction to the claim of Goodwin, Land and Osman ('55) that the same total concentration of carotenoids occurs in aerobic and anaerobic cultures of *R. spheroides.* The contradiction is only apparent, however, since the conditions of supposed aerobic cultivation used by Goodwin *et al.* were in fact wholly inadequate to ensure the free access of air to the population. This conclusion can be drawn from their statement of the methods employed: "Aerobic cultures (100 ml) were grown in Erlenmeyer flasks (250 ml). All cultures were shaken vigorously by hand twice a day" (Goodwin, Land and Osman, '55). Except in the earliest stages of development, only a tiny fraction of the population in such a culture could be truly "aerobic." It has, of course, long been known that slant cultures and colonies of *R. spheroides* and *R. rubrum* which are allowed to develop under aerobic conditions in the dark eventually show appreciable pigmentation; but here again, the environment is virtually anaerobic, since the outer cells act as a biological barrier against oxygen diffusion into the mass of the population. Such qualitative observations show, however, that pigment synthesis can be resumed, *even in the absence of light,* when the oxygen tension is reduced to very low levels. The pattern of pigmentation in a colony that is growing aerobically in the dark shows very clearly the necessity of limited oxygen access for the development of pigment. When just large enough to be seen with the naked eye, such a colony is completely colorless. As its size increases, pigment begins to accumulate in the central region, while the cells in the growing periphery — still exposed to a relatively high oxygen tension — remain colorless. A study of the kinetics of pigment synthesis in the dark at very low partial pressures of oxygen might be productive of interesting results, but owing to the technical difficulties we have not yet attempted it.

The effect of air on the carotenoids of the wild type of R. spheroides

When air is introduced into a photosynthetically grown culture of the wild type of *R. spheroides,* the color of the cells changes fairly rapidly from brown to red (French, '40; van Niel, '44). This change was first investigated chemically by van Niel ('47), who showed that it is caused by a change in the ratio of the two carotenoid pigments. In anaerobic cultures, the ratio yellow pigment/red pigment is usually about

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2:1, whereas in cultures exposed to air it may fall to 1:3. Van Niel found that when fully grown anaerobic cultures were aerated for 16 hours, the disappearance of the yellow pigment was exactly balanced by the formation of the red one since the total carotenoid content of the population remained constant. He therefore concluded that the yellow pigment can be directly converted to the red one under the influence of oxygen. The change appears to be irreversible. Goodwin, Land and Osman ('55) have recently questioned the interpretation offered by van Niel to account for these changes, and have suggested instead that there is no direct biosynthetic link between the two pigments, the effects of air on the pigment ratio being caused by preferential synthesis of the red carotenoid and destruction of the yellow one. They appear to have reached this conclusion largely on the basis of the observation that in a supposedly aerobic (but actually semi-anaerobic) culture, the yellow pigment disappeared without a concomitant formation of the red one. Although they state that this change took place in a growing population, examination of their data reveals that the disappearance of the yellow pigment began at least 5 days after the population had entered the stationary phase. Accordingly, they were studying pigment changes in a senescent and probably moribund population, and their findings have no relevance to the problem of the yellow-red conversion as studied by van Niel, who employed conditions which were relatively normal from the physiological standpoint.

Incidental to the study of the effects of air on pigment synthesis, we have followed the yellow-red conversion in exponentially growing populations of *R. spheroides,* and our findings are in good accord with those reported by van Niel for resting populations. Since air almost completely arrests carotenoid synthesis by *R. spheroides,* its effect on the yellow-red ratio in growing cells can be studied with little interference from net synthesis. Some typical data, taken from the growth experiment shown in fig. 10, are presented in figure 13.

The changes in the carotenoid content of the culture through time have been plotted on an arithmetic scale rather than a logarithmic one, in order to reveal the striking complementarity of the changes in yellow and red carotenoid that follow the introduction of air. In the first 30 minutes of exposure to air, there was a very sharp fall in the amount of yellow carotenoid, and a correspondingly abrupt rise in the rate of synthesis of the red one: the relation is not stoichiometric, since net synthesis was still taking place at an appreciable rate. During the ensuing hours of aeration, while net synthesis was negligible, the amount of yellow carotenoid continued to diminish and synthesis of the red pigment was maintained, although at a rate far below that observed during the first 30 minutes of exposure to air, and below that during the preceding period of anaerobic growth. Upon return of the culture to anaerobiosis rapid net synthesis was resumed,

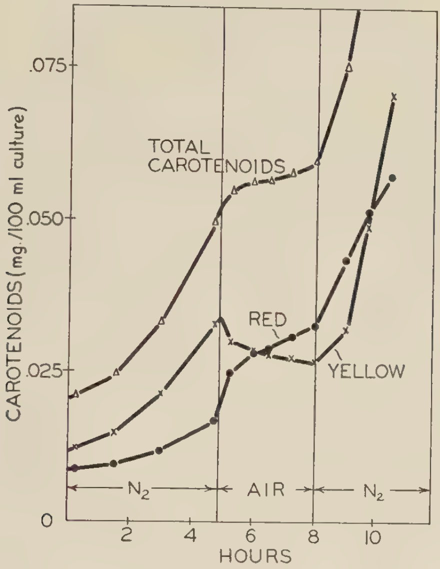

Fig. 13 The influence of air on the carotenoid pigments of the wild type of *Rhodopseudomonas spheroides*. Data from the growth experiment shown in figure 10.

and the predominance of the yellow pigment was re-established by preferential synthesis. These observations are fully in accord with the predictions which follow from van Niel's hypothesis of a direct and irreversible yellow-red conversion, but necessitate some highly implausible kinetic assumptions if one attempts to explain them in terms of two distinct biosynthetic pathways.

The kinetics of porphyrin excretion by R. spheroides

When cultures of *R. spheroides* which have entered the stationary phase after growth in anaerobiosis and light are centrifuged, the supernatant fluid has a reddish-brown color. This is caused by a substance or mixture of substances having the characteristic spectrum of porphyrins with a sharp Soret peak at 405 mμ in water, and showing strong pinkish-orange fluorescence under ultraviolet light. The process of excretion can be followed roughly by measurement of light absorption at 405 mμ on the clarified culture medium after sedimentation of the cells, and its kinetics were examined in several growth experiments. As shown in fig. 14, no trace of extracellular porphyrin appears while a culture under photosynthetic growth conditions is still growing exponentially and synthesizing chlorophyll. Immediately after the population enters the stationary phase (an event which coincides with the cessation of chlorophyll synthesis), rapid accumulation of extracellular porphyrin begins, and continues for several hours if anaerobic conditions and illumination are maintained. This phenomenon has been observed only in anaerobic cultures of *R. spheroides* grown in the light, never in aerobic ones. In our experience, it does not occur at all with *R. rubrum.*

In *Corynebacterium diphtheriae,* iron depletion is known to elicit the excretion of porphyrins (Pappenheimer, '47). This cannot be the cause of the porphyrin excretion which we have observed with *R. spheroides,* since the arrest of growth results from depletion of the organic hydrogen donor: in photosynthetic cultures whose growth was limited at different final levels by the concentration of malate supplied, entry of the population into the stationary phase was invariably followed by porphyrin excretion.

During the course of these experiments we observed that in the supernatant liquid derived from samples of cultures taken shortly after porphyrin excretion had begun, the optical density at 405 mμ was not stable, but instead increased markedly with time. The rate and magnitude of this change, measured on a sample kept in the dark at room temperature, is shown in figure 15. Figure 16 shows the evolution of the

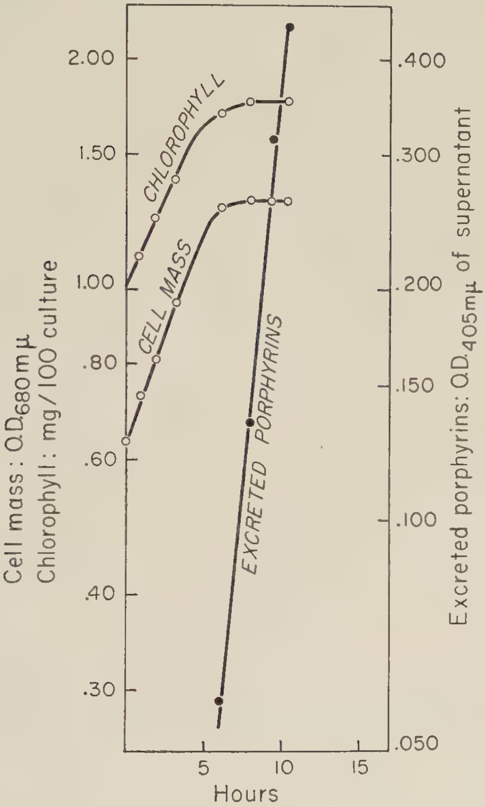

Fig. 14 The kinetics of porphyrin excretion by a culture of the green mutant of *Rhodopseudomonas spheroides* grown anaerobically in the light.

absorption spectrum of the same sample during the period of increasing optical density at 405 mμ. The quantity of cells in such supernatant culture fluids was negligible, since they had been centrifuged at gravitational fields sufficiently high to cause an essentially complete sedimentation of the bacteria; and no precipitable material could be detected upon treatment with trichloroacetic acid. Hence it seems probable that the material excreted by the cells is not a porphyrin, but an extremely labile precursor capable of giving rise to porphyrins by spontaneous decomposition.

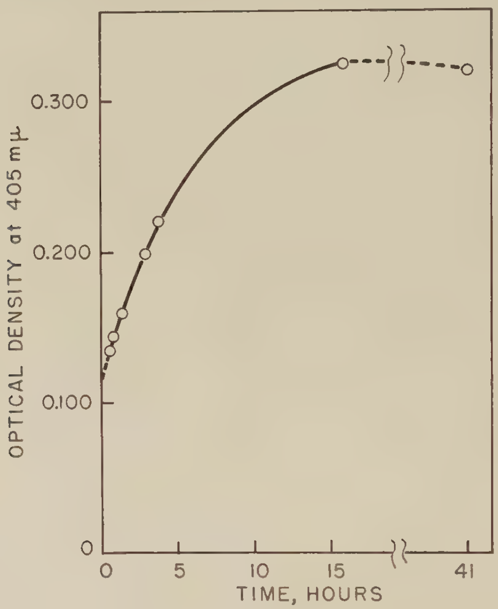

Fig. 15 Optical density changes at 405 mμ in the supernatant liquid from a culture of *R. spheroides* in the early stationary phase. Zero time represents the time of harvesting: the first reading was taken some 20 minutes later, after removal of the cells by centrifugation. The supernatant was kept at room temperature in the dark.

The phenomena described above are undoubtedly related to the excretion of porphyrins by resting cells of *R. spheroides,* which has been studied in considerable detail by Lascelles ('54, '56). She has made the plausible suggestion that this excretion is in some way associated with the synthesis of bacteriochlorophyll. With resting cells, she observed that porphyrin excretion required light and was inhibited by air, even in the presence of light, an observation which checks well with our findings on the environmental conditions required for the appearance of extracellular porphyrins in cultures.

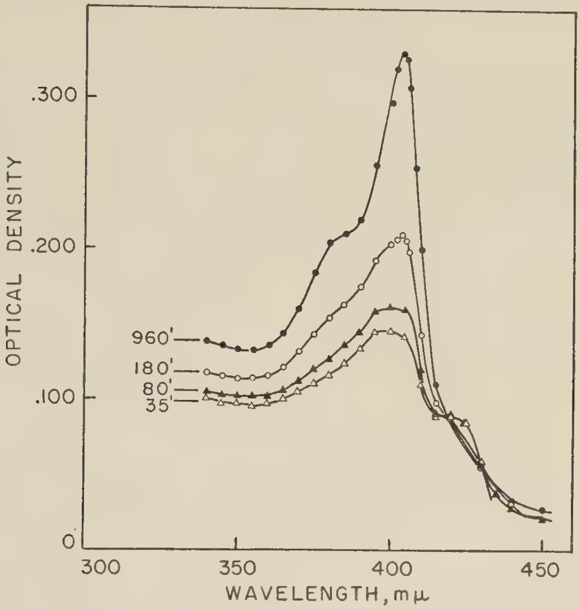

Fig. 16 Changes in the absorption spectrum of the supernatant liquid from a culture of *R. spheroides* in the early stationary phase. The times indicated beside each spectrum are those which had elapsed since removal of the sample from the culture. After sedimentation of the cells, the supernatant liquid was kept at room temperature in the dark.

It should be noted that these conditions are also necessary, if not sufficient, conditions for the synthesis of chlorophyll. The fact that extracellular porphyrin accumulation in a culture begins only after growth and chlorophyll synthesis have ceased suggests that the cell may begin to excrete porphyrins when it no longer has available all the biosynthetic precursors necessary for the complete synthesis of the chlorophyll molecule, but is still able to carry out the early steps (i.e., up to the porphyrin stage) with the materials at its disposal. Since in our experiments the chlorophyll content of the cells remains perfectly stable throughout the period of porphyrin excretion, it is most improbable that the porphyrin is formed by the degradation of pre-existing bacteriochlorophyll.

The regulation of photosynthetic pigment synthesis: an interpretation

The kinetic studies reported in this paper show that even during photosynthetic growth, the manufacture of photosynthetic pigments by *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* is highly autonomous: pigment synthesis can occur in the absence of general cellular growth, and *vice versa.* It is also evident that the syntheses of chlorophyll and carotenoids are broadly coupled, even though these pigments are entirely different in chemical structure and do not, so far as is known, share a common pathway of biosynthesis.

Under anaerobic conditions, the cell responds to changes of light intensity by temporarily suppressing or accelerating pigment synthesis. An increase of light intensity, if of sufficient magnitude, causes an immediate suppression of pigment synthesis: a decrease of light intensity immediately accelerates the rate of pigment synthesis. Under aerobic conditions, on the other hand, pigment synthesis is permanently and almost completely suppressed at atmospheric oxygen tension, only carotenoid manufacture continuing, at an extremely low rate. It is tempting to postulate that the cell possesses a single physiological mechanism for the regulation of pigment synthesis. Such a mechanism must be capable of responding both to light intensity and to oxygen, a fact which should provide some clues as to its nature. We shall, therefore, approach the problem of interpretation by considering what systems in the cell could be expected to fall under the influence both of light and oxygen, and to respond in broadly similar fashion either to the introduction of oxygen or to a sudden increase in light intensity.

The essential features of photosynthetic and respiratory metabolism by non-sulfur purple bacteria are shown schematically in figure 17. The primary photochemical reaction in these organisms is generally considered today to be identical with that in green plants — a photolysis of water to yield hydrogen and hydroxyl radicals. The hydrogen radical furnishes the reducing power required for the reductive step in the synthesis of organic material from CO_2. The path of CO_2 fixation is here schematized in simplest terms as: $CO_2 \rightarrow (CH_2O)$, the latter symbol standing for synthesized cell material. The details of these reactions are not relevant to the argument which will be developed. According to current notions, the [OH] radical arising from the primary photochemical reaction serves as a terminal oxidant for the electrons which are produced during the stepwise dehydrogenation of the organic hydrogen donor. This implies that [OH] is coupled to the whole system of electron transport by means of a (probably specific) terminal oxidase, which may be the photooxidase described by Kamen and Vernon ('54). The extraneous hydrogen donor is oxidized to CO_2, but can also serve in part as a direct source of cell material, since it furnishes organic fragments capable of undergoing assimilation, symbolized in the diagram as (CH_2O). Oxygen enters

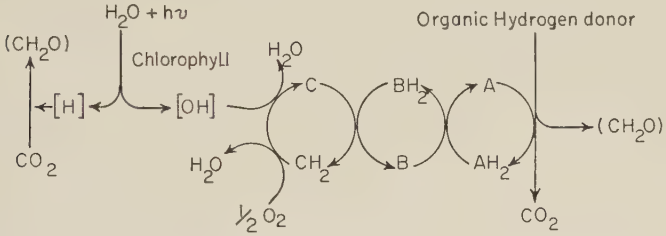

Fig. 17 A schematic diagram illustrating the essential features and interconnections of photosynthetic and respiratory metabolism in non-sulfur purple bacteria.

the metabolic picture as an alternative terminal oxidant for electrons derived from the organic hydrogen donor: like [OH], it is connected to the transport system by a specific terminal oxidase, the dark oxidase of Kamen and Vernon ('54).

From this resumé, it can be seen that one system in the cell — namely, the chain of carriers involved in electron transport — is strongly influenced both by light and oxygen. When a steady flow of electrons from the organic hydrogen donor is passing through this system, the steady state levels of oxidation of its members will be determined by the rate at which electrons are removed by the reduction of [OH] or O_2. Hence either a sudden increase of light intensity (which will increase the rate of formation of [OH]) or the introduction of air will shift the carrier system towards a more oxidized state, as shown diagrammatically in figure 18. This supposition has recently been verified experimentally by Chance and Smith ('55), who have studied changes in the level of oxidation of the cytochrome system in living cells of *R. rubrum* upon exposure to light or oxygen. On the other hand, a decrease in light intensity or a shift from aerobiosis to anaerobiosis will tend to put the carrier system in a more reduced state (fig. 19).

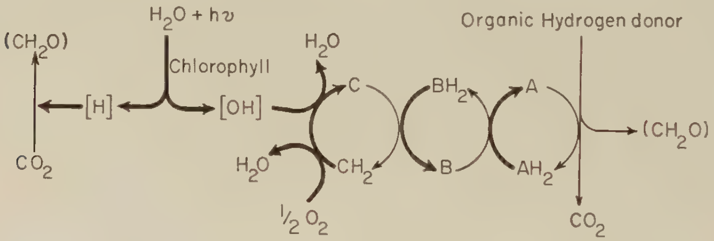

Fig. 18 The effect of introducing air or of increasing light intensity on the intracellular oxidation level of the electron transport system of non-sulfur purple bacteria. The favored reactions are indicated by heavy arrows.

All our observations on the kinetics of photosynthetic pigment synthesis can be explained on the physiological level by the assumption that the rates of both chlorophyll and carotenoid synthesis are inversely related to the state of oxidation of some member of the carrier system. When this carrier is largely reduced, synthesis proceeds at a high rate; as it becomes increasingly oxidized, the rate of pigment synthesis declines, reaching zero at a certain critical ratio of oxidized: reduced carrier. It must be assumed that this critical ratio is slightly larger for carotenoid synthesis than for chlorophyll synthesis, in order to explain the respects in which these synthetic processes respond differently to environmental changes; namely, the continuation of carotenoid synthesis at a very low rate in aerobiosis, where chlorophyll synthesis is totally suppressed; and the slightly faster resumption of carotenoid synthesis under anaerobic conditions following suppression of photosynthetic pigment synthesis by a sudden increase of light intensity.

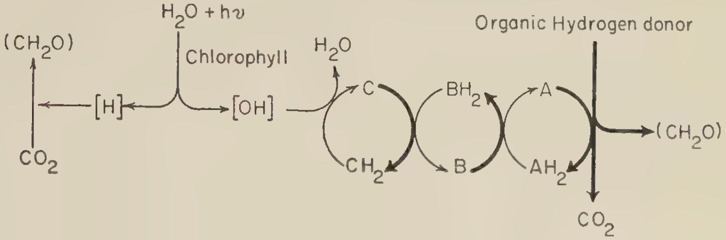

Fig. 19 The effect of establishing anaerobiosis or of decreasing light intensity on the intracellular oxidation level of the electron transport system of non-sulfur purple bacteria. The favored reactions are indicated by heavy arrows.

Let us now consider how this hypothesis can be used to explain in detail the sequence of events which follows a sudden increase of light intensity in a population that is growing under anaerobic conditions, as illustrated in figure 5 A. The inoculum in this experiment had been growing under steady state conditions at a fixed light intensity, the differential rates of pigment synthesis being stabilized by the oxidation level of the regulatory carrier, which in turn was stabilized by the light-controlled flux of [OH]. As a consequence of the sudden increase of light intensity, the flux of [OH] increased, and the regulatory carrier, along with the other components of the respiratory chain, became oxidized to a new level, considerably above the critical level for both chlorophyll and carotenoid synthesis. The rates of both syntheses therefore fell abruptly to zero. Since the population was growing at an undiminished rate, the photosynthetic pigment content per cell began to fall, and as a consequence the flux of [OH] in each cell also began to fall from the maximum attained immediately after the change of light intensity. In turn, the oxidation level of the regulatory carrier began to fall, first reaching the critical level for carotenoid synthesis, which then resumed, and afterwards reaching the critical level for chlorophyll synthesis, which resumed some time later. With the resumption of photosynthetic pigment synthesis, a partial brake was placed on the decline in the flux of [OH] per cell, and consequently on the fall in the oxidation level of the regulatory carrier. However, the oxidation level of the regulatory carrier would necessarily continue to decline until a steady flux of [OH] could be maintained, and at a constant light intensity, this condition requires that the rate of synthesis of the photosynthetic pigments be exactly in balance with their rate of dilution by growth, i.e., that the steady state level of pigment synthesis be reached. A similar argument can be constructed to account for the reverse changes which follow a sudden fall of light intensity, illustrated in figure 5 B.

It remains to show how other facts about pigment synthesis find their explanation in terms of this hypothesis. One is the extremely high differential rate of pigment synthesis, practically reaching infinity, that is observed in the first phase of recovery of bleached cells when they are placed under conditions of obligatory photosynthesis, i.e., anaerobiosis and light. In such cells, the extreme dilution of the photosynthetic pigment system results in a very feeble flux of [OH]. Since the other alternate terminal oxidant for the carrier system, molecular oxygen, is excluded, all the components of the carrier system will be thrown into a very highly reduced state, since the pressure of electrons from the organic hydrogen donor will still be operating as effectively as ever. This reduced state of the carrier system, which according to the hypothesis should cause photosynthetic pigment synthesis at maximal differential rate, can only be alleviated by the synthesis of more pigment, which permits an increase in the flux of [OH]. The changes in the differential rates of pigment synthesis actually observed in such recovery experiments (fig. 9) are fully in accord with expectations.

The powerlessness of light to maintain photosynthetic pigment synthesis under aerobic conditions is very simply explained: oxygen causes an oxidation of the regulatory carrier to a level above that at which chlorophyll synthesis can occur, and as long as it is present at the tension necessary to maintain this oxidation level, the resumption of pigment synthesis is impossible. Lastly, we must consider the observation that pigment synthesis can occur in the absence of light when the oxygen tension of the environment is very low. Presumably at extremely low oxygen tensions the dark oxidase is not saturated, and hence the oxidation level of the regulatory carrier can fall below the critical value at which chlorophyll synthesis begins. Accurate kinetic analysis of this pigment synthesis in the dark would provide one critical means of testing the validity of our general hypothesis, which predicts that the differential rate of pigment synthesis and the activity of the dark oxidase should respond in similar fashion to changes in the partial pressure of oxygen.

Although in general the regulatory mechanism for pigment synthesis employed by *R. rubrum* and *R. spheroides* has obvious adaptive value for the organism, there are two conditions under which it operates non-adaptively: namely, in suppressing pigment synthesis when the cell is exposed simultaneously to light and air; and in eliciting pigment synthesis by the cell at low partial pressures of oxygen in the dark. These two non-adaptive responses can be regarded as necessary consequences of the means of regulation which the cell has adopted, and represent a relatively small biological price to pay for a mechanism of great physiological simplicity and sensitivity.

Many of the possible ways of testing the validity of our general hypothesis involve considerable difficulties, but we have already explored one which is relatively simple, experimentally speaking. Under anaerobic conditions at a fixed light intensity, the depletion of CO_2 should reduce the flux of [OH], since the [H] radicals derived from the primary photochemical split, being incapable of performing their normal function in the reducing step of the path of CO_2 fixation, should recombine with [OH] as shown in figure 20. Hence it can be predicted that depletion of CO_2 at a fixed light intensity should have the same effect as a dimming of the light — i.e., stimulation of the differential rate of photosynthetic pigment synthesis — since both environmental changes should increase the state of reduction of the regulatory carrier. It should be recalled that during the photometabolism of non-sulfur purple bacteria, CO_2 is simultaneously produced from the hydrogen donor and fixed in the dark reactions of photosynthesis; whether a net evolution or a net fixation of CO_2 takes place depends on the oxidation level of the organic hydrogen donor. With malate, a relatively oxidized substrate, there is a considerable net evolution of CO_2, and hence malate cannot be employed in experiments where one wishes to block the CO_2 fixation reaction by depletion. Accordingly, a more reduced hydrogen donor, succinate, was used for these experiments. A culture of the green mutant of *R. spheroides* was prepared in the usual basal medium containing 0.02 M ammonium succinate as

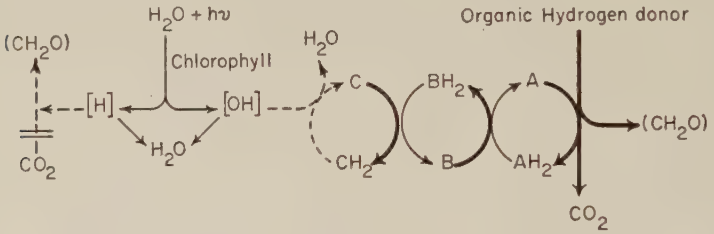

Fig. 20 The effect of reducing CO_2 fixation on the intracellular oxidation level of the electron transport system of non-sulfur purple bacteria. The favored reactions are indicated by heavy arrows, and the hindered reactions by broken arrows.

hydrogen donor, and incubated anaerobically at a fixed light intensity. The aeration mixture at the start of the experiment was 95% N_2–5% CO_2. When steady state conditions of growth and pigment synthesis had been reached, the gas mixture used for aeration was changed to 100% N_2. At the moment of change the system contained considerable bound CO_2, and at first growth continued at a fairly high rate. However, as the reserve of bound CO_2 was used up, the growth rate fell, eventually becoming negligible. At this point, aeration with 95% N_2–5% CO_2 was resumed, and rapid growth began once more. Owing to the major changes in growth rate which occurred during the experiment, a plot of growth and chlorophyll synthesis on a time scale is confusing. The results are presented instead as a plot of the increase in the amount of chlorophyll in relation to the increase in cell mass (fig. 21); the slope of the resulting curve thus describes directly the differential rate of chlorophyll synthesis. After the removal of CO_2 from the

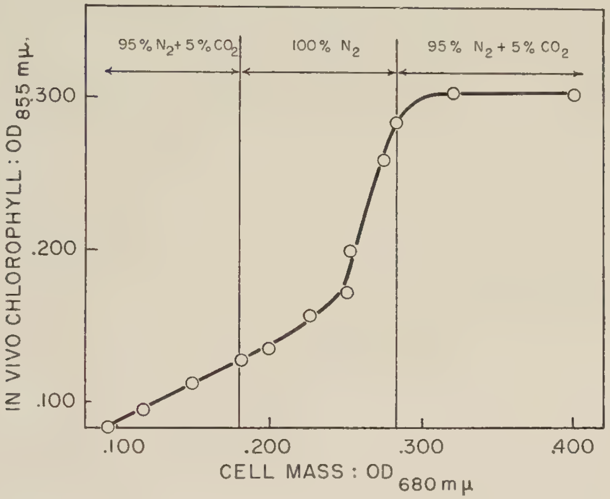

Fig. 21 The differential rate of chlorophyll synthesis at a fixed light intensity by the green mutant of *Rhodopseudomonas spheroides,* growing with succinate as hydrogen donor and in the atmosphere either of 95% N_2 – 5% CO_2 or 100% N_2 as indicated on the graph. Chlorophyll estimated from the light absorption *in vivo* at 855 mμ, corrected for scattering.

aeration system, the differential rate of chlorophyll synthesis at first changed little, since the culture was still well supplied with bound CO_2. Thereafter, as the growth rate started to decline, indicating approaching depletion of CO_2, the differential rate of chlorophyll synthesis rose sharply. The reintroduction of CO_2 restored growth and at the same time completely suppressed chlorophyll synthesis, since during the period of CO_2 starvation the cells had built up their chlorophyll content to a level far above the steady state level characteristic of the light intensity employed. The results of this experiment accordingly agree well with prediction.

The hypothesis which has been outlined in the preceding paragraphs is a highly satisfactory one as far as it goes, which is to explain the regulation of pigment synthesis on a physiological level. Obviously, it leaves all the important biochemical and structural questions unanswered; but the experimental analysis which we undertook was not designed or intended to answer questions on a biochemical level. It may nevertheless be worthwhile to conclude by indicating the main biochemical and structural problems which now require elucidation. The first of these is to establish the nature and position in the respiratory chain of the postulated regulatory carrier. The second is to examine the behavior of the chromatophores (the submicroscopic cytoplasmic units which are the sites of the photosynthetic pigment system in the cell) during changes in the pigment content of the cell. By analogy with the behavior of plant chloroplasts, the chromatophores may well be capable of growing at a rate different from that of the cell, and it remains possible that the regulation of pigment content involves primarily a regulation of the rate of chromatophore growth relative to that of cellular growth. The third problem is the search for a biochemical mechanism whereby the level of oxidation of the postulated regulatory carrier could serve to control pigment synthesis, either by controlling directly the operation of the two biosynthetic pathways or, more indirectly, by controlling chromatophore growth.

SUMMARY

1. Methods have been developed for the quantitative study of chlorophyll and carotenoid synthesis by non-sulfur purple bacteria under controlled conditions of growth.

2. The kinetics of photosynthetic pigment synthesis have been examined in two species, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum.* These organisms respond in broadly similar fashion to environmental factors which affect pigment synthesis. Under anaerobic conditions in the light, the differential rates of photosynthetic pigment synthesis are inversely related to light intensity. When the light intensity is suddenly changed, the population adjusts its pigment content by a transient suppression or stimulation of pigment synthesis. An increase of light intensity causes suppression, a decrease, stimulation. Under aerobic conditions, whether in light or dark, chlorophyll synthesis is completely suppressed and carotenoid synthesis nearly so. Visual observations suggest, however, that photosynthetic pigment synthesis can resume even in the dark at very low partial pressures of oxygen; as yet, this phenomenon has not been studied kinetically.

4. On the basis of these observations, a general hypothesis to explain the mechanism by which the cell regulates synthesis of the photosynthetic pigments has been constructed. This hypothesis assumes that the rate of chlorophyll and carotenoid synthesis is governed by the state of oxidation of a carrier in the electron transport system: when this carrier is largely reduced, pigment synthesis proceeds at a high rate; as it becomes more oxidized, the rate of pigment synthesis diminishes, eventually reaching zero at a critical level of oxidation of the regulatory carrier.

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EFFECT OF POTASSIUM ON ELECTROPLAX OF ELECTROPHORUS ELECTRICUS [1]

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NINE FIGURES

INTRODUCTION

During the last decade important progress has been made in the elucidation of the events that determine bioelectric potentials. It has been shown that the action potential is produced by a net influx of sodium and that this movement follows the electrochemical gradient established across the cell membrane (Hodgkin and Katz, '49). This gradient is maintained, at least partly, through the active extrusion of sodium (Krogh, '46; Levi and Ussing, '48; Steinbach, '51; Hodgkin and Keynes, '55a). A comprehensive picture of the electric potentials of an axon and many of its physiological characteristics in terms of differential distribution and movement of ions have been developed (Hodgkin and Huxley, '52b).

On the other hand, the origin of the resting potential remains an obscure problem. It is well known that many investigators believe that this potential is produced by the diffusion of potassium. Objections have been raised to this hypothesis (Grundfest et al., '54; Grundfest, '55; Hitchcock, '55) and the recent demonstration that the influx of potassium is due to an active transport across the membrane makes it even more

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questionable (Shanes, '51; Hodgkin and Keynes, '55a and '55b).

For several years attempts to clarify the events underlying the electrical potentials in the electroplax have been made in this laboratory. The results obtained show that these cells are similar in their physiological and pharmacological characteristics to striated muscles (Altamirano et al., '53a and '55b). The question as to the role of sodium and potassium ions is therefore important. Keynes and Martins-Ferreira ('53) previously studied this problem. With regard to potassium, they found that an excess of this ion in the saline solution sometimes produced a small depolarization of the cell and an equivalent decrease of the amplitude of the spike. This depolarization was frequently irreversible. Their preparation was not quite adequate for these studies, as was discussed by the authors. For this reason it was considered useful to investigate again this problem with the preparation developed in this laboratory (Altamirano, '55a). During these studies a number of experiments were performed testing the electrical resistance of the membrane of the electroplaque, and the changes induced by excess or lack of potassium in the saline solution. These investigations are the subject of this paper.

METHODS

The methods were described and discussed in detail in a previous publication (Altamirano, '55a). In summary, the preparation may be described as a single row of electroplax mounted on two silver wires. The upper and lower surfaces of each cell were in direct contact with the saline. Successive electroplax were usually less than 0.6 mm apart and were separated by extra-cellular tissue. The total thickness of the preparation measured about 1 mm or less. The whole preparation could be either immersed in saline of any desired composition or maintained in air. When the action potential was measured with the preparation kept in the air, both amplitude and duration were greater than when recorded in saline solution.

The standard saline had the following molar composition: NaCl 0.190; KCl 0.005; $MgCl_2$ 0.0015; $CaCl_2$ 0.003; sodium phosphate buffer 0.005.

Solutions were prepared with an excess or lack of potassium; details are described with the experimental results. The saline in the last two experiments listed in table 1 contained no sodium, this ion being completely replaced by an isoosmotic concentration of sucrose.

RESULTS

1. Effect of an excess of potassium on the resting and action potentials measured with the electroplax immersed in saline. The resting potential of axons and striated muscle fibers decreases reversibly when the concentration of potassium in the environment is increased above the normal. The action potential is also diminished or completely inhibited according to the concentration of potassium used (Hodgkin, '51b). Similar observations were performed by Keynes and Martins-Ferreira ('53) with electric tissue of electric eel. Their results, as mentioned before, were irregular. They suggested that some of the irregularities may be attributed to the fact that the concentration of potassium was increased in one face of the cell only. If one side of the electroplaque is depolarized, a difference of potential will appear across the cell and a flow of current established that could damage the membrane. This situation was avoided in the experiments here described, in which the concentration of potassium was increased in the whole environment of the cell.

The potassium-rich solutions contained 5, 10, 15 or 20 times the concentration used in the standard saline solution (0.005 M). In some cases potassium chloride was added to the saline solution and, therefore, the osmotic pressure of the solution was increased. In other experiments the concentration of sodium chloride was reduced by an amount equal to the potassium salt added, i.e. the osmotic pressure was kept unchanged (table 1). All these solutions produced effects that

TABLE 1

EXPERIMENT NO.	DATE	CONTROL					CONC. OF KCl IN TESTED SOLUTION (M)	TIME AFTER EXPOSRUE TO SOLUTION TESTED (MIN.)	EFFECT OF SOLUTION TESTED					TIME AFTER WASHING WITH NORMAL SALINE (MIN.)	RECOVERY				
		Resting potential, mV.	Action potential, mV.	Resistance at rest $\frac{\text{Ohm}}{\text{cm}^2}$	Resistance after activity, Ohm/cm²	Maximum rate of rise of the spike, V/sec.			Resting potential, mV.	Action potential, mV.	Resistance at rest $\frac{\text{Ohm}}{\text{cm}^2}$	Resistance after activity, Ohm/cm²	Maximum rate of rise V/sec.[1]		Resting potential, mV.	Action potential, mV.	Resistance at rest $\frac{\text{Ohm}}{\text{cm}^2}$	Resistance after activity, Ohm/cm²	Maximum rate of rise of the spike, V/sec.
1	9-23-55 [B]	88	154	5	21.2	309	0 [2]	32	144	175	13.6	21.2	331	43	95	156	. .	. . .	231
2	9-26-55 [B]	94	147	2.9	9.9	285	0 [2]	60	142	168	5.0	9.9	289	55	96	147	. .	. . .	257
3	9-27-55	88	134	4.5	13.3	238	0 [2]	24	130	172	7.6	13.3	238	35	84	132	. .	. . .	170
4	9-21-55	90	127	. .	. . .	152	0.025 [2]	16	50	76	. .	. . .	160	52	88	138	. .	. . .	180
5	9-22-55	84	140	4.6	12.5	162	0.025 [2]	15	60	109	2.7	12.5	184	57	82	142	. .	. . .	172
6	7-13-55	82	118	. .	. . .	142	0.025	7.5	58	76	. .	. . .	200	55	80	114	. .	. . .	110
7	7-14-55	83	115	5.4	13.1	100	0.025	12	55	82	2.9	12.2	124	38	81	115	4.8	13.1	98
8	6-15-55	80	145	4.1	8.3	. . .	0.05	9	44	40	1.7	9.1	. . .	58	80	146	3.2	8.3	. . .
9	7- 7-55	80	120	3.0	5.2	144	0.05	12	44	52	1.6	5.2	185	53	84	124	. .	. . .	. . .
10	10-14-54	86	120	. .	. . .	. . .	0.05	31	52	56	. .	. . .	. . .	72	68	90	. .	. . .	. . .
11	10-19-54 [3]	86	140	. .	. . .	. . .	0.05	36	46	56	. .	. . .	. . .	139	82	140	. .	. . .	. . .
12	10-21-54 [3]	88	140	. .	. . .	. . .	0.05	48	48	0	. .	. . .	. . .	. . .	. .	. . .	. .	. . .	. . .
13	11-22-54 [3]	72	152	7.5	. . .	. . .	0.05	13	44	0	4.5	. . .	. . .	123	80	161	7.5	. . .	. . .
14	11-23-54 [3,4]	80	144	3.3	10.5	. . .	0.05	12	54	0	1.2	10.5	. . .	126	80	146	3.8	11.8	. . .
15	11-29-54	84	150	5.8	11.3	. . .	0.05	17	46	0	2.3	10.2	. . .	114	80	135	5.8	. . .	. . .
16	9-23-55 [A]	88	146		. . .	187	0.05 [2]	12	47	0	. .	. . .	225	101	90	140	. .	. . .	242
17	9-26-55 [B]	90	142	3.6	11.5	152	0.05 [2]	15	53	0	2.0	11.5	160	80	89	138	. .	. . .	200
18	11-10-54 [5]	86	122	. .	. . .	. . .	0.075	10	52	0	. .	. . .	. . .	24	72	100	. .	. . .	. . .
19	11-24-54 [3]	81	142	5.1	12.4	. . .	0.1	5	45	0	1.4	9.7	[7]	104	82	140	7	13.1	. . .
20	7-15-55	83	136	5.6	16.4	173	0.1	8	46	0	2.1	11.6	230	74	82	134	5.1	16.4	216
21	7-18-55	83	142	7.6	15.2	230	0.1	6	45	0	4.0	12.9	[7]	80	82	145	. .	. . .	266
22	6- 9-55 [A 6]	77	133	. .	. . .	140	0.12 [2]	8	22	0	. .	. . .	[7]	73	74	130	. .	. . .	136
23	6- 9-55 [B 6]	75	128	5.2	14.9	157	0.12 [2]	5	26	0	3.3	19.7	[7]	85	80	124	. .	. . .	174

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[1] Greatest rate of rise recorded after the addition of testing solution.

[2] All solutions tested were hyperosmotic by the amount of potassium chloride added, except those marked [2] that were isoosmotic (see text page 81).

[3] All data given were obtained with the preparation immersed in saline solution, except those of experiments marked [3] which were obtained with the electroplaque in air.

[4] Five hundred microns per milliliter of procaine were added to the solution 38 minutes before the exposure of the preparation to the potassium-rich solution (see fig. 5 and text page 83).

[5] One hundred microns per milliliter of procaine were added to the solution before the exposure of the preparation to the potassium-rich solution.

[6] The sodium of the saline solution was completely replaced by an isoosmotic amount of dextrose.

followed the same pattern. The course of a typical experiment performed with 5 times the normal concentration is shown in figure 1. The magnitude of the resting potential (open circles) and action potentials (closed circles) are indicated in millivolts (ordinates). The amplitude of the spike and the resting potential decreased rapidly after the excess of potassium was added, reaching its lowest value in about 10 minutes; in contrast, the overshoot diminished only 5 mV and in other experiments remained approximately the same. The maximal depolarization was reached within the first 7 minutes in two of the other three experiments performed with 0.025 M potassium chloride. The electric potentials

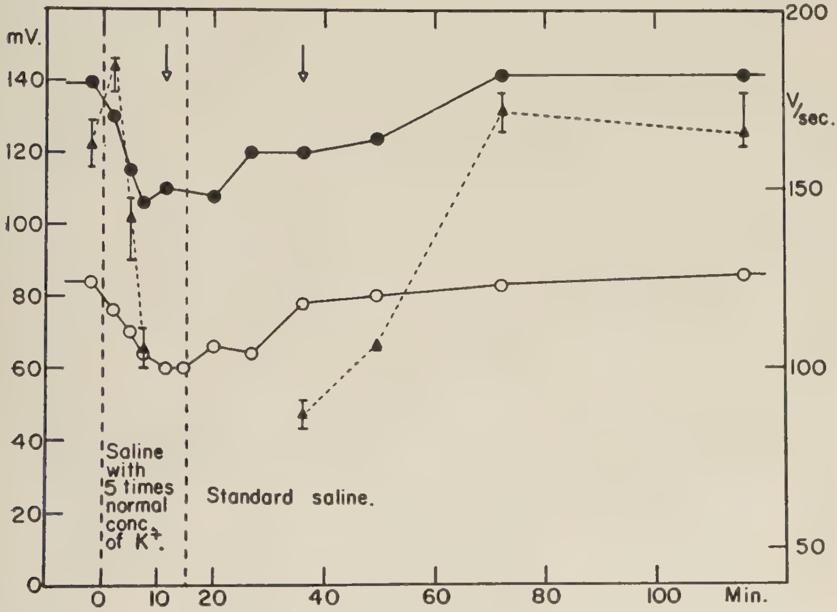

Fig. 1 Effect of 0.025 M potassium chloride on the electrical potentials of the electroplax. Ordinates at left: magnitude in mV of the action potential (closed circles) and resting potential (open circles). Ordinates at right: maximal rate of rise of the spike in V/sec. (triangles). Abscissae: time in minutes. Between the two vertical broken lines, the preparation was immersed in a solution containing 0.025 M potassium chloride. At the time indicated by the second broken line, the preparation was washed repeatedly with standard saline solution. The propagation of the spike was blocked between the two arrows; only local responses were obtained.

slowly recovered to the previous level after the preparation was washed with saline, but more than 60 minutes were necessary for a complete recovery. The first arrow indicates when the spikes ceased to be propagated along the cell; the second arrow shows when propagated action potentials were again obtained. Therefore, the magnitudes of the local responses measured across the active membrane are plotted between the arrows.

The maximal rate of rise of the propagated action potential has also been plotted in figure 1. When the action potential ceases to be propagated the rate becomes proportional to the strength of the stimulus and has no definite value. Therefore, it has not been plotted between the arrows. The excess of potassium caused an increase of this rate during the first minutes of exposure in all the experiments performed. It seems that this increase was more pronounced when the potassium-rich solution was hyperosmotic (table 1). Later, when the propagation of the spike began to be impaired, this rate markedly decreased. It is interesting to point out that this increase of the rate of rise has not been observed when other depolarizing substances, for example acetylcholine or some of its derivatives, are used. The latter substances produce only a slowing of the rising phase of the spike (Altamirano, '55b).

The increased rate of rise produced initially by an excess of potassium may reappear transitorily after washing the preparation with saline, as shown in figure 1.

It may be pointed out that the absolute value of the maximal rate of rise is about 10 to 20% higher than the magnitudes given in figures 1 and 2 and table 1. This difference arises from the long time constant of the recording system used (about 100 microsec.). Since the same pair of electrodes was used throughout an experiment, this inaccuracy does not reflect on the significance of the relative changes described.

Figure 2 illustrates a similar experiment performed with a solution containing 20 times the normal concentration of potassium (0.1 M). A rapidly increasing depolarization followed the exposure to the potassium-rich solution. Initially the rate of rise of the spike increased despite the diminished amplitude of the action potential. Soon the spike ceased to be propagated (first arrow) and only local activity could be elicited for a short period. This eventually disappeared. A small local activity is difficult to measure, because the artifact of the strong stimulus introduces an important error. The usual procedure, of subtracting the artifact produced by an anodic pulse from the response to a cathodic stimulus, is not adequate; for the resistance of the membrane is markedly different with regard to the direction of the current flow when activity is elicited, as will be discussed later. The error in the measurement of small local activity may be as high as 50% or more.

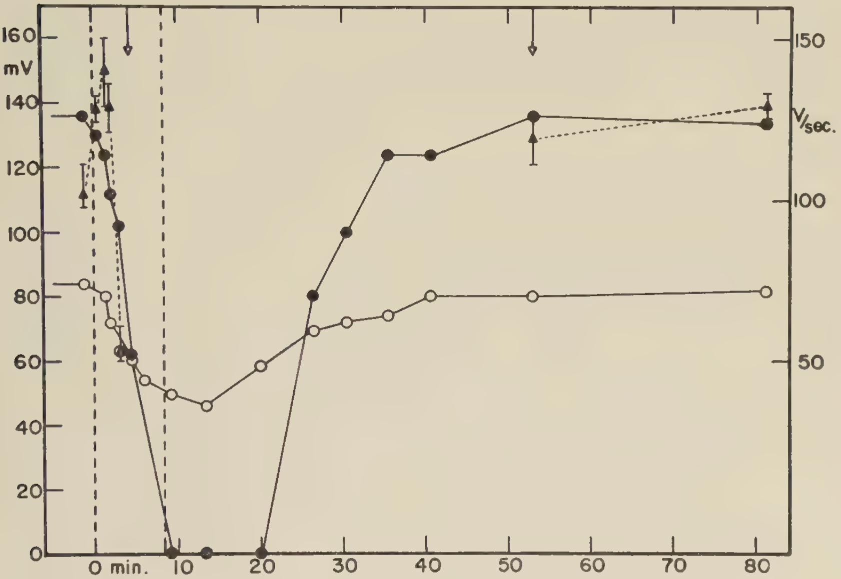

Fig. 2 Effect of 0.1 M potassium chloride on the electrical potentials of the electroplax. Ordinates, abscissae and symbols as in figure 1. Between the vertical broken lines, the preparation was exposed to a saline solution containing 0.1 M potassium chloride; after the second broken line it was washed repeatedly with standard saline solution.

Therefore, it is possible that small responses were still present when the activity indicated in the figure is zero. As in the preceding figure, the resting and action potentials slowly returned to the original level after washing the preparation with saline.

A summary of the results obtained with experiments performed with potassium-rich solutions is presented in table 1. The general findings presented in this table were also obtained in other experiments which are not included, either because they were performed under different experimental arrangements or because chemical substances were used prior to the exposure of an excess of potassium.

In some of the experiments listed in table 1 data on the rate of rise of the spike are omitted because the time scale of the recordings was too slow to allow reliable measurements of this rate. Some recordings were done with the preparation in air and others with the preparation immersed in the saline solution, as indicated in the table. The magnitude of the action potential is usually larger when the former method is used, as discussed previously (Altamirano, '55a), but otherwise the experiments are comparable.

It has been claimed that in squid axon (Curtis and Cole, '42) myelinated frog nerve fiber (Huxley and Stampfli, '51) and some striated muscle cells (Ling and Gerard, '50) the depolarization produced by an excess of potassium has approximately the magnitude predicted by the Henderson equation. This agreement has been considered as a demonstration that the resting potential is some kind of diffusion potential dependent on the extracellular and intracellular concentration of potassium. The results summarized in table 1 have been plotted in figure 3 in a way more adequate to visualize what should be the depolarization obtained if the resting potential depends exclusively on the potassium concentration. An important difficulty in tracing the curve shown in figure 3 arises from the fact that the internal concentration of potassium of the electroplaque is not accurately known. Davson and

Lage ('53) found 176 and 187 mM of potassium per kilogram of water respectively in the large electric organs of two different electric eels. In this laboratory (unpublished) the Large Organ of three electric eels were analyzed and 175, 190 and 163 meq/kg of potassium per killogram intracellular water were found. The average of the 5 figures is 178 meg/kg int. water of potassium. If the concentration of potassium inside the cell is taken as 178 meq/kg water, the values of the resting potential as predicted by the simplified Henderson equation are shown by the full line in figure 3. When the external concentration of potassium is 0.005 M the average value of the resting potential is 83 mV. The range of variation is also indicated in this figure. Keynes and Martins-Ferreira ('53) obtained an average magnitude somewhat higher, i.e. 86 mV. When the external concentration of potassium is

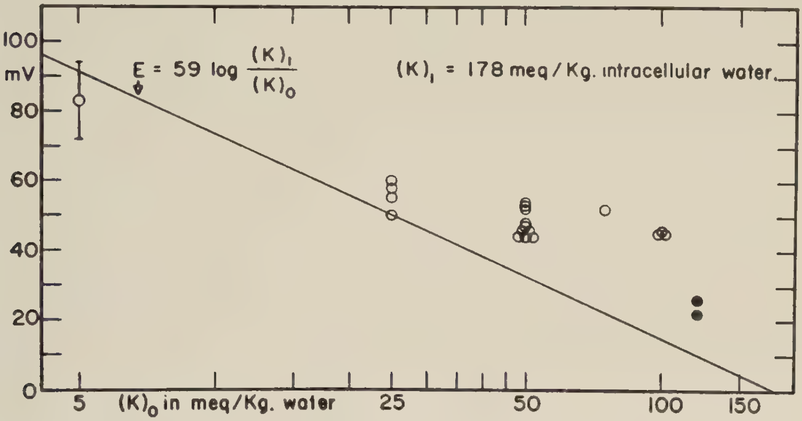

Fig. 3 Effect of an excess of potassium on the resting potential of the electroplax. Ordinates: resting potential in mV. Abscissae: concentration of potassium in the saline solution in meq/kg water (logarithmic scale). The solid line represents the value of the resting potential as predicted by Henderson's equation. The intracellular concentration of potassium is assumed to be as indicated in the figure (see text). The resting potential considered normal (at 0.005 M extracellular concentration of potassium) is the average of the values given in table 1. The range of variation is also indicated. All the experiments of table 1 have been individually plotted in this figure (open circles). The closed circles correspond to the last two experiments of table 1.

raised to 5 times the normal, the depolarization observed is somewhat less than the value predicted by the Henderson equation, but considerable discrepancies exist when higher concentrations are used (fig. 3).

It may be argued that the depolarization recorded during exposure to solutions containing 10 to 20 times the normal concentration of potassium may not be the maximum because the diffusion of the ion to the membrane of the electroplaque was hindered by the remaining extracellular tissue. The extracellular concentration of potassium in the immediate vicinity of the membrane may have been somewhat lower than the value assumed when the resting potential was determined for the last time. This argument may be questioned. The upper and lower boundaries of the preparation and, therefore, of each electroplaque, were in direct contact with the saline. The layer of extracellular tissue that separates the electroplaques and covers the other two sides of the cells is at most 1 mm in thickness and also is in contact with the saline by its upper and lower surfaces. The extracellular tissue contains 96% water and its electrical resistance is the same as the resistance of the solution with which it is at equilibrium. The mobility of its constituent ions is, therefore, probably the same as in a saline of similar composition. The concentration of KCl in the extracellular tissue at different depths after exposure to the potassium-rich solution was calculated by means of a formula derived by Hill ('29).[2]

The preparation was considered as a thin sheet of tissue of about 1 mm in thickness, exposed on both sides to the solution. The diffusion coefficient of potassium chloride in a 0.1 M solution was taken as 9.6×10^{-4} cm^2/min. for curve A (Vinograd

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[2] The equation used is the following:

$$\frac{C_r}{C_s} = 1 - \left[\frac{4}{\pi} e^{-\frac{k\pi^2 r}{4b^2}} \sin \frac{\pi}{2}\frac{x}{b} \right]$$

in which C_r = molar concentration of potassium in the extracellular tissue at depth $b = x = 0.05$ cm; C_s = molar concentration of potassium in the saline solution; k = diffusion coefficient of potassium chloride in cm^2 min.$^{-1}$; t = time of exposure of the preparation to the saline.

and McBain, '41) and half this value for curve B. Figure 4 shows a curve in which the ordinate represents the concentration at the center of the preparation of potassium chloride as a fraction of that at the surface after different times of exposure to the potassium-rich solution (abscissa). It may be seen that the extracellular space of the preparation will be practically at equilibrium with the saline within 7 minutes.

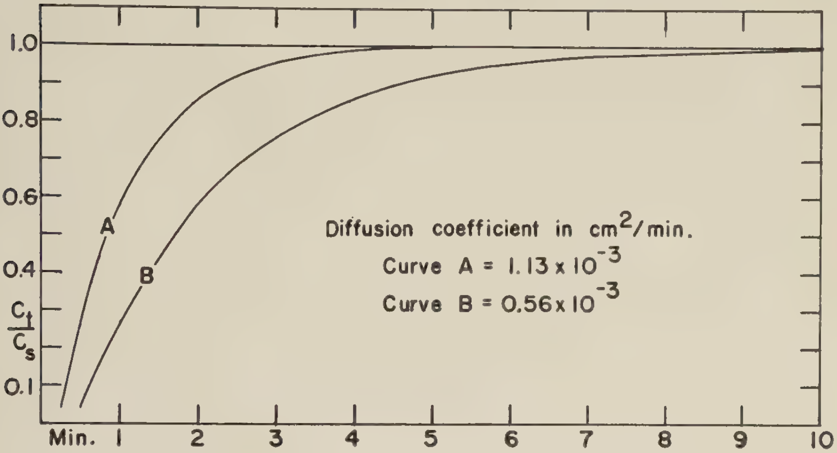

Fig. 4 Time required for the extracellular tissue of the preparation to be at equilibrium with the potassium concentration of the saline. The two curves were calculated according to the equation derived by Hill ('29). The value obtained by Vinograd and McBain ('41) in a 0.1 M solution of potassium chloride was used for the diffusion coefficient of potassium chloride in curve A; in curve B half this value was used. Ordinates: ratio of the concentration of potassium at the center of the extracellular space with the concentration of the ion in the saline. Abscissae: time in minutes elapsed from the moment at which the saline solution to be tested was added.

The curve presented refers to the diffusion of potassium chloride into a solution lacking this salt. Since the extracellular space initially contained at least 0.005 M of KCl, the equilibrium probably was reached before that period of time. In agreement with this statement it was observed that in many experiments the resting potential did not continue decreasing after the first 7 minutes of exposure to the excess of potassium.

The addition of sufficient solid potassium chloride to the saline solution to increase its partial molar concentration to 0.1 M (20 times the normal), causes a marked increase of the total osmotic concentration of the solution. The osmotic pressure of the potassium-rich solution (20 times the normal concentration) is about 1.4 times the osmotic pressure of the standard saline. In order to have an idea of the role played by this excessive osmotic concentration on the resting potential, some experiments were performed in which the osmotic concentration of the solution was increased by addition (a) of sucrose, and (b) of solid sodium chloride. It was observed that the resting potential increases about 10 mV when the osmotic pressure of the saline increases by a factor of 1.4. If these results are applicable to the situation in which the increased osmotic pressure is produced by an excess of potassium chloride, then the depolarization observed in the latter case should be less than that predicted by equations like that of Henderson. However, the figure of table 1 shows that this is not the case. Within the limits tested the depolarization is about the same with isoosmotic or hyperosmotic solutions.

The last two experiments of table 1 show the action of a potassium-rich solution in absence of sodium. The depolarization obtained was significantly larger than in the presence of the standard sodium concentration. These two experiments suggest that the depolarization produced by potassium is not due to an influx of sodium, i.e. to an indiscriminate increase of the membrane permeability to all ions.

The results described so far were obtained by stimulation of a limited region of the electroplaque immersed in the saline, with a square pulse of 0.1 to 0.5 m sec. duration. When the same preparation is maintained in air (Altamirano, '55a), it is possible to excite simultaneously the whole innervated membrane. The current density sufficient for such a stimulation may be evaluated and the resistance of the membrane may be calculated. Since the whole innervated membrane is activated simultaneously, the action potential produced is non-propagated. These action potentials have a higher amplitude, are longer, and their rising phase is steeper than the spikes recorded when the preparation is immersed in saline (Altamirano, '55a). The differences mentioned arise fundamentally from the important change in resistance of the extracellular media when the preparation surrounded by the saline is compared with the preparation maintained in air.

The changes produced by an excess of potassium on the action and resting potential recorded with the preparation in air were similar to the changes observed when the preparation was kept under saline during the recordings. Since they followed the general pattern described in the previous section, they will not be described here. The experiments described in the next two sections refer to results obtained with the cells maintained in air during the performance of the measurement analyzed.

2. *Stimulation with long square pulses (5 to 22.5 m sec.) with the preparation in air.* When long square pulses are used for stimulation, it is preferable to record the cell potentials with the preparation in air because a more uniform gradient of potential is attained across the cell and the density of the current utilized is easily measured (Altamirano, '55a). In figure 5A are shown a series of traces of the displacement of the transmembrane potential produced by pulses of 22.5 m sec. duration. Only the first part of each record is illustrated. The numbers at left indicate the stimulating current used in milliamperes per cubic centimeter. The zero trace corresponds to a spike elicited by a 0.3 m sec. stimulus, therefore during the development of activity there was no extraneous electric field superimposed upon the membrane. A cathodic pulse of 5.2 mA/cm^2 produced a displacement of the transmembrane potential of 18 mV, but since it was subthreshold no activity ensued. The threshold stimulus depolarized the cell to about 20 mV; 5.9 mA/cm^2 were necessary to produce this effect. The general characteristics of the activity obtained when the stimulating current is maintained during the development of the action potential has been described elsewhere (Altamirano, '55a). After the spike subsides, the transmembrane

potential decreases to a lower value. This potential is a function of the current utilized (traces 5.9 to 11.7). Very strong pulses cause a depolarization that may be of the same magnitude or higher than the action potential. In this case the activity of the cell does not add a potential to the record

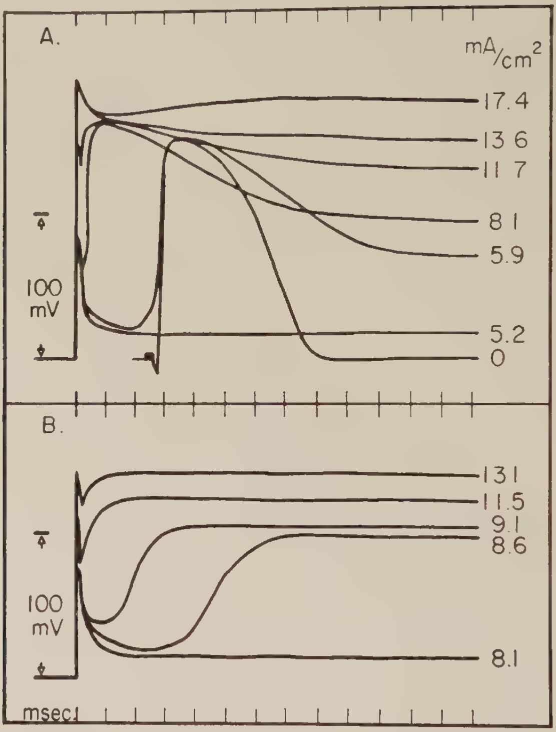

Fig. 5 Effect of an excess of potassium on the electrical characteristics of the electroplax. In A and B the electroplax was stimulated by a cathodic pulse which lasted 22.5 msec. The strength of the pulses is indicated at the right of each trace in mA/cm². The amplitude calibration corresponds to 100 mV. A: responses of the electroplaque immersed in standard saline solution. The stimulating pulse lasted only 0.3 msec. in trace O. Between A and B the preparation was immersed 38 min. in a solution of 500 μg/ml of procaine. This compound did not change the resting potential (80 mV) or the amplitude of the action potential (144 mV) when elicited by stimulation of the whole innervated membrane. Then a solution containing procaine and 0.05 M potassium chloride was added and the preparation tested again 12 min. later (B). The resting potential decreased to 54 mV and the strongest stimulus used did not elicit a response, except the changes of the transmembrane potential shown.

(trace 17.4). It has been mentioned previously (Altamirano, '55a) that this situation should occur when the electrochemical potential of sodium is the same inside and outside the cell, and therefore could provide information on the "equilibrium" potential of sodium.

The set of traces of figure 5B were obtained in the same preparation as the records of figure 5A, but after 10 minutes of exposure to a solution containing 10 times the normal concentration of potassium. The resting potential decreased from 80 to 54 mV and no responses could be elicited by short pulses, whatever their strength. Previous to the exposure to the excess of potassium the preparation was immersed for 38 minutes in a solution containing 500 μg/ml of procaine. The latter compound did not modify the resting potential or the stationary spike and did not prevent the effect of potassium. This conclusion can be verified by comparing the results of experiments of table 1 with those in presence of procaine.

The records of figure 5B raise the interesting question whether the changes of the transmembrane potential there illustrated are equivalent to the action potential elicited in a cell under normal conditions. It is evident that these changes have a definite threshold, and provided the superimposed electric field is long enough, the transmembrane potential attains a value directly dependent on the intensity of the electric field. For example, 8.1 mA/cm^2 depolarized the cell by 10 mV for as long as the pulse was applied. A larger current (8.6 mA/cm^2) caused, after a short latent period, a progressive change of the transmembrane potential. The depolarization just sufficient to initiate this change of the transmembrane potential (fig. 5B) has the same magnitude as the threshold depolarization necessary to elicit the spike in the electroplax immersed in standard saline, i.e. about 20 mV (figs. 5A and B). An important property of this change in transmembrane potential is that it is not self-regenerative as the normal spike is. In the experimental conditions described, it existed only for as long as the superimposed electric field was present. Figure 6 illustrates this view. A square pulse of the same magnitude but of increasing duration was applied in all the records. In A its duration is insufficient to produce the phenomenon under discussion. The progressive development of the full transmembrane potential is shown in B, C, D and E. It is also shown how the transmembrane potential broke down as soon as the superimposed field was cut off. The potential returned to the resting level following an exponential curve.

The ratio of the displacement of transmembrane voltage to the current in figure 5A (after the spike has subsided) has the same constant value as in figure 5B. This result suggests, as will be discussed in the next section, that a progressive change of the transmembrane potential similar to that shown in figure 5B occurs in the electroplax immersed in standard saline when subjected to the action of an electric field, although in the latter case the presence of the spike complicates the initial part of the records.

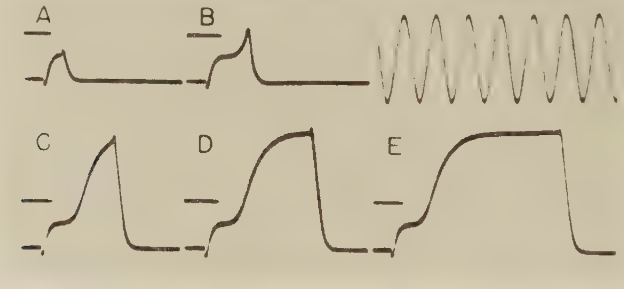

Fig. 6 Effect of an excess of potassium on the transmembrane potential of the electroplax. The preparation was immersed 14 min. in a solution containing 10 times the normal concentration of potassium (0.05 M). The resting potential decreased from 72 to 52 mV and no response could be elicited except the changes of the transmembrane potential shown in the records A to E. The strength of the stimulus was the same from A to E (6.3 mA/cm^2) but its duration was increasingly prolonged. Cal.: 100 mV and 1000 cycles.

3. *Effect of an excess of potassium on the resistance of the membrane.* The experimental arrangement used with the preparation kept in air permits the simultaneous measurement of the current density used for stimulation and the

displacement of the transmembrane potential produced by this current. Henceforth the resistance of the membrane may be calculated directly from Ohm's law. As may be seen in table 1, the absolute resistance of the membrane is rather low if compared with the resistance of other biological membranes. This problem was discussed in a previous publication (Altamirano, '55a). It was mentioned that the area of the innervated membrane of each cell is measured as if it were a plane sheet; however, it has so many digitations and irregularities of the surface, that its real area may be higher by a factor of hundred or more. Consequently, the real current density may be smaller by a similar factor and the absolute resistance per square centimeter one hundred or more times higher. Therefore, the indicated absolute values of resistance should not be considered as a measure of the actual value. On the other hand, the changes produced in the resistance of a given cell by different agents in relatively short experiments (1 to 2 hours) are a correct measure of the relative changes of resistance within the error of the measuring instruments, since it is not probable that the area of the cells will change much.

Figure 7 shows the results obtained in one experiment performed with 5 times the standard concentration of potassium. The signs of the voltage and the current are applied in the way previously described (Altamirano, '55a); the current that determines a depolarization is considered positive (outwardly directed) and the displacement of the transmembrane potential produced has a positive sign (the inside of the cell is more positive). Anodic currents and the hyperpolarization produced by them are plotted with a negative sign. The arrows at right indicate when the depolarization reached threshold and elicited an action potential. The open circles to the left of these arrows represent the values obtained when the membrane was at rest; it is evident that the resistance of the membrane behaved as an ohmic resistance and that there was no rectification provided there was not activation of the membrane. The curves to the right of the arrow will be analyzed later. The addition of a solution containing 5 times the normal concentration of potassium decreased within 5 min. the resistance from 4.6 ohm/cm^2 to 2.7 ohm/cm^2, i.e. a decrease of about 40%. This change in resistance was accompanied by a reduction of the resting potential from 84 to 60 mV. Washing the preparation with saline produced the recovery of the resting and action potential to the original magnitude. A summary of the results is presented in table 1; an important decrease of the resistance was observed in all the experiments performed. An excess of potassium produced a similar effect on axons of *Carcinus maenas* (Hodgkin, '47a). Apparently there is no relation between the amount of potassium in the solution and the reduction of the resistance, but it must be pointed out that the experiments were not always performed under the same conditions. For example, the resistance was measured after

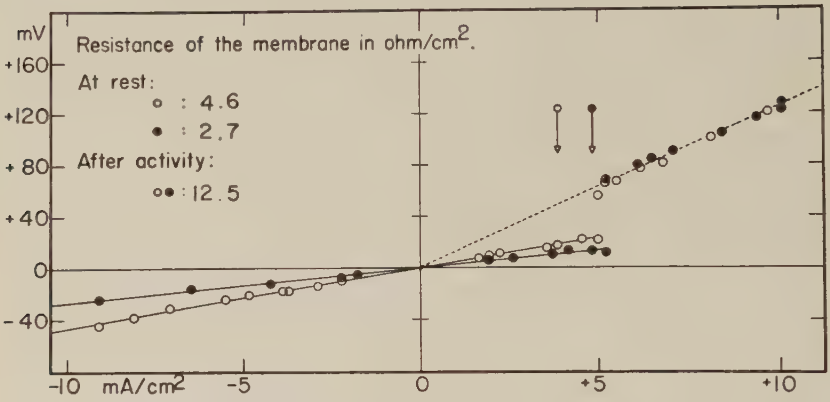

Fig. 7 Effect of an excess of potassium on the resistance of the membrane. The ordinates indicate the displacement (mV) of the transmembrane potential produced by the curent (mA/cm^2) as shown by the abscissae. The displacement of potential is taken as positive when the electroplaque is depolarized; the current flowing outwards is considered positive. The arrows indicate when the depolarization reaches threshold. The points to the left of each arrow correspond to the membrane at rest; to the right to values obtained after activity (for details see text and Altamirano, '55). Open circles: experimental values obtained with the preparation in standard saline. Closed circles: values obtained after exposure to saline containing 0.05 M potassium chloride.

different periods of time of exposure to the excess of potassium that were always shorter in the case of the concentrated solutions. In the latter cases a longer exposure may have caused a higher reduction. However, within the range tested the decrease in resistance does not appear to be a linear function of the external concentration of potassium.

The line of resistance to the right of the arrow in figure 7 is more complex as to its significance. The transmembrane voltage plotted is the potential measured across the membrane after the initial activity, for example the action potential, has subsided. It may be seen in this figure that there is a linear relationship between the magnitude of the displacement of the transmembrane potential and the current that caused the displacement of the transmembrane potential. This problem was discussed in a previous paper (Altamirano, '55a), and it was concluded that this part of the curve is a measure of the resistance of the membrane during the decaying phase of the spike. The resistance is usually about two or three times higher than that at rest. In figure 7 it is clearly seen that despite the excess of potassium decreasing the resistance of the membrane at rest, the resistance after activity is not significantly modified.

The procedure followed in plotting the points to the right of the arrows after exposure of the cell to an excess of potassium needs some additional comments. As stated above, in this condition no activity may be elicited when the electroplaque is stimulated with short pulses (for example at an external concentration of potassium of 0.05 to 0.1 M). It, therefore, may not seem justified to describe this resistance as resistance "after activity." However, when the cell is stimulated with long suprathreshold pulses, a progressive change of the transmembrane potential takes place as discussed in connection with figure 5B. In figure 7 the maximal values reached by the transmembrane potential have been plotted against the current necessary to produce this potential drop. The modifications of the transmembrane potential illustrated in figures 5A and B are obviously the result of a change of the electric characteristics of the membrane that takes place after a critical depolarization has been reached. This phenomenon may be explained in two ways: either the critical depolarization determines a progressive increase of the membrane resistance reaching a maximum value that is proportional to the strength of the applied field, or an electromotive force, similar in origin to the action potential, is built across the membrane during this prolonged stimulation. The equivalent circuit of the experimental arrangement used has been described previously (Altamirano, '55a).

Figure 7C of the mentioned paper shows clearly that only an increase of R_M (resistance of the membrane) or B_M (electromotive force developed by the membrane) or both may explain a finding similar to the results here described.

The hypothesis that a new electromotive source is built across the membrane, seems improbable. As explained above, the change of the transmembrane potential is non-regenerative and lacks, therefore, one of the most important characteristics of the action potential. Also the ratio of the displacement of the transmembrane potential to the current applied has the same constant value whether determined in the normal cell or after exposure to an excess of potassium as shown in figure 7. The same is true with regard to the absolute magnitude of the displacement of potential. It thus appears highly probable that the same underlying process is present in both situations. It was shown that in the normal cell in this phase there is an increase of the membrane resistance and no evidence was found of the existence of an electromotive source similar to the process that causes the spike. Results of experiments performed with sodium-free solutions, to be published separately, strongly support this assumption.

In summary, the exposure to an excess of potassium causes an important decrease of the resistance of the membrane at rest. In this situation, an adequate stimulus elicits, even when the spike is completely abolished, a progressive increase of the resistance of the membrane. The maximal value attained is equal to the resistance measured during the falling phase of the spike in the cell before exposure to potassium. As shown in figure 5B, the time at which this maximal value is reached is a function of the intensity of the applied field. When the latter depolarized the cell by about 140 mV (trace 131) the maximal membrane resistance was reached in about 1 msec. It may be seen in figure 5A that the decaying phase of the normal spike in the same preparation (trace 0) started about 1 msec. after the initiation of the latter. During this action potential the membrane was depolarized by about 150 mV. In all the experiments analyzed completely the same correspondence was found. Therefore, it may be suggested that the decaying phase of the spike is the conveyance of this increase of membrane resistance that obviously is a measure of a decreased ionic permitivity.

4. *Action of potassium-free saline.* It has been observed in different tissues that the lack of potassium in the external media causes a small increase of the resting potential (Hodgkin and Katz, '49; Hodgkin and Keynes, '55b; Huxley and Stampfli, '51; Ling and Gerard, '50). A striking increase was obtained in the electroplaque that usually begins within the first minute after the immersion of the preparation in the potassium-free solution (fig. 8). The resting potential still slowly increased one hour after the exposure started. It is difficult to evaluate the actual concentration of potassium outside the membrane, for a net outflux of potassium must exist which will tend to increase the extracellular concentration of the ion in the immediate vicinity of the membrane. Figure 8 shows also that the action potential increased for a while in a magnitude equal to the increase of the resting potential, that is the overshoot remained unmodified; this should be expected if the action potential is solely due to a net influx of sodium. The spike thus reached quite high values. The amplitude of the action of potential of experiment no. 1 (table 1) was 214 mV when measured with the preparation in air after 30 minutes of exposure to potassium free saline. It was not measured in air in the experiment illustrated in figure 8, but the absolute magnitude of the spike was prob-

ably similar since it measured 180 mV with the electroplaque immersed in the saline.

After a while, however, the action potential ceased to be propagated (first arrow in fig. 8) and the overshoot of the local response diminished. It is clear that the membrane underwent important changes in its physiological properties when the period of exposure to potassium-free saline was prolonged. The maximal rate of rise of the spike decreased before the block of propagation was established. A similar decrease of this rate was observed when the cell was exposed to an excess of potassium, even as small as 5 times the standard external concentration. The mechanisms that determine the change in permittivity to sodium of the membrane were obviously affected in both cases. Again the complete recovery from this condition took a much longer time than that necessary for the establishment of the original ionic concentrations across the membrane.

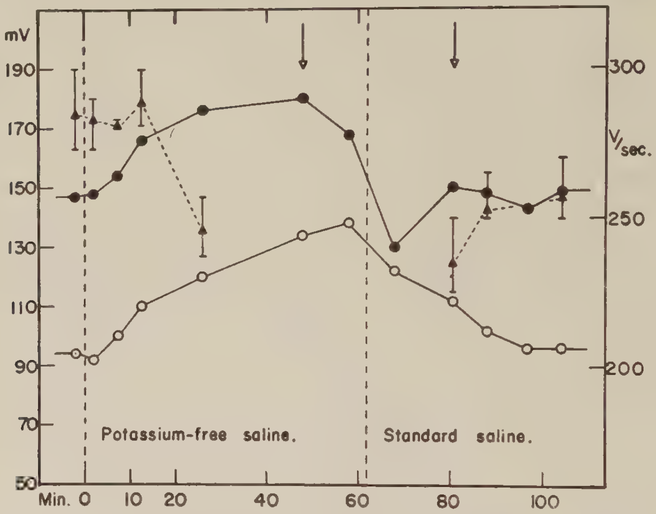

Fig. 8 Effect of potassium-free saline on the electric potentials of the electroplaque. Ordinates, abscissae and symbols as in figure 1. Between the two broken lines the preparation was immersed in a potassium-free saline. The preparation was repeatedly washed with standard saline after the second broken line.

The potassium-free solution produced similar effects on the resting potential and action potential as a maintained anelectrotonus. In both cases the action potential increased by the same magnitude as the resting potential and the propagation of the spike was blocked when the hyperpolarization passed a given level. Before reaching a hyperpolarization that had a blocking effect, an important decrease of the maximal rate of rise of the action potential was noticed. This observation suggests that the increase of permeability to sodium of the membrane during the rising phase takes place at a slower rate. This does not seem to be consistent with the observation of Hodgkin and Huxley ('52a) that a prolonged anelectrotonus inhibits the "sodium inactivation process." However, the experimental conditions of the two sets of observations are different and therefore not readily comparable.

The resistance of the membrane at rest increased when the saline solution did not contain potassium. The partial potassium-conductance of the membrane might be evaluated if the membrane could be comletely deprived of this ion, which as mentioned earlier is not feasible. Furthermore, as shown in figure 8, after a long exposure to a potassium-free saline, the membrane lost its normal physiological properties, and it is doubtful if the information obtained in this period can be applied to the normal membrane. However, figure 9 and table 1 support the view that the lack of potassium in the external solution approximately doubles or triples the resistance of the membrane at rest. On the other hand, the resistance of the membrane during the falling phase of the spike was not modified (fig. 9). The longer the exposure to potassium-free saline, the longer the increment of resistance. This also applies to the increase of the resting potential. In the light of the calculations of figure 4 the concentration of potassium should be reduced to a small and constant amount within the first 10 minutes.

5. *Effect of anodic polarization on the block of conduction caused by excess of potassium.* During the performance of these experiments, it was observed repeatedly that anodic

polarization increased markedly the amplitude of the action potential when it has been previously inhibited by an excess of potassium. This effect was also noticeable after the use of other compounds that determine a reduction of the resting potential of the cell, as acetylcholine or some of its derivatives. In the electroplaque, the unblocking action of the anelectrotonus was not dependent on the presence of any recognizable "slow" potential, as observed in frog nerve by Lorente de Nó ('47). The anelectrotonus also increased the magnitude of the spike in the electroplaque immersed in standard saline solution. Similar effects were recorded during the hyperpolarization produced by the potassium-free saline. It may be suggested that the anelectrotonus increased the amplitude of the activity inhibited by an excess of potassium through the same mechanisms by which any hyperpolarization increases the amplitude of the spike in a normal cell (Hodgkin and Huxley, '52b). Therefore, the local responses obtained

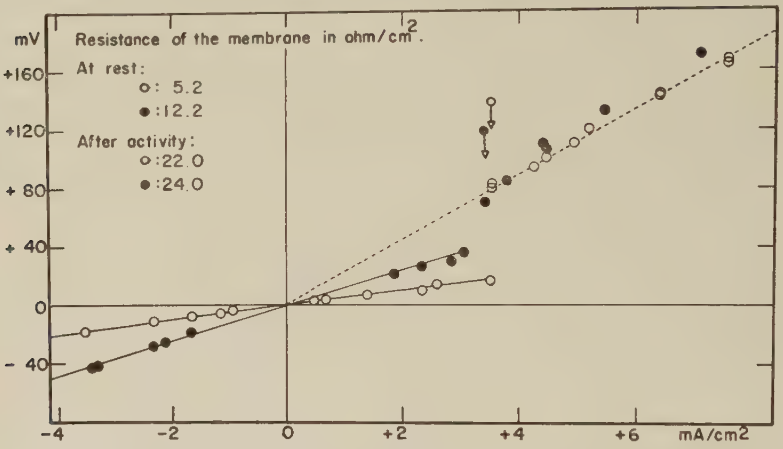

Fig. 9 Effect of a potassium-free solution on the resistance of the membrane of the electroplax. Ordinates and abscissae as in figure 7. Open circles: Resistance of the membrane with the preparation in standard saline solution. Closed circles: resistance of the membrane after exposure for 30 min. to potassium-free saline solution. The line of resistance after activity corresponding to the closed circles is not drawn; the value indicated in the figure corresponds to the best approximation.

in a segment of an axon blocked by potassium will increase in magnitude when an anelectrotonus is applied. This increase of the magnitude of the local response, if large enough, may be sufficient to stimulate the distal segment. Now the wave of activity may propagate again across the blocked region.

The anelectrotonus increased the magnitude of the activity inhibited by an excess of potassium not only during its duration, but if long enough (10 to 20 msec.) for 20 or more msec. after it ended. Therefore, as also observed in frog nerve by Lorente de Nó ('47), its unblocking properties outlasted the existence of the applied electric field. A significant increase of the excitability of the preparation was evident for 20 or more msec. in a normal electroplaque after a long anelectrotonus. If this increase of excitability was strong enough, it determined what has been classically known as the ''break excitation'' at the anode (unpublished). A similar increase of excitability was demonstrated in the electroplaque after its activity was depresed by an excess of potassium. When the response is local, an increase in excitability usually determines an increase of the amplitude of the response because a larger area of membrane is activated by a given stimulus. It may be argued, therefore, that the delayed unblocking action of the anelectrotonus is mediated by the increase of the cell excitability. The same given stimulus will cause larger local responses which may excite the distal segment of the blocked axon.

DISCUSSION

It is apparent from the results described that an excess or lack of potassium produces effects on the electroplax essentially similar to those in nerve or striated muscle fibers. However, there are several quantitative differences between its action on the latter tissues and on the electroplax which are interesting.

In the first place, there is a large discrepancy between the magnitude of the depolarization caused by the excess of potassium and the depolarization predicted by Henderson's or similar equations, which is too great to be attributed to experimental errors. The afore mentioned discrepancy is even greater than is observed in frog muscle ('50). In nerve fibers the resting potential apparently varies according to the external concentration of potassium within the limits predicted by these equations (Hodgkin, '51; Curtis and Cole, '42; Huxley and Stampfli, '51). This agreement has been considered as a demonstration that the resting potential is determined by the ratio of the intracellular to the extracellular concentration of potassium. The above argument seems incorrect because the same results should be expected whether the resting potential is a potential produced by the diffusion of potassium or whether it is determined by any other mechanism, as can be easily shown. Moreover, the findings obtained with electroplax are apparently in disagreement with the foregoing hypothesis. Also, Hodgkin and Keynes ('55a and '55b) have shown that the influx of potassium is an active process when its external concentrations are low. Henderson's equation seems therefore inadequate for explaining the potassium distribution.

The effect of an excess of potassium on the action potential of the electroplax follows a more conventional pattern. At low external concentration (less than 0.25 M) it reduces the magnitude of the spike in a value equal to the decrease in resting potential and the overshoot remains unchanged. Apparently the mechanisms that determine the change in permeability to sodium are not yet significantly modified. If the external concentration exceeds 0.025 M, the normal behavior of the membrane is completely disrupted, the overshoot is abolished and only small, graded responses are elicited that eventually disappear in time. The mechanisms by which an excess of potassium prevents the elicitation of the spike are not known, but it is obvious that important biochemical changes take place in the membrane in this situation. This view is suggested by the extremely long time needed for the complete recovery of the membrane after exposure to an excess of potassium, a time longer by a factor of 6 or more than the time that theoretically should be necessary for a reversion to the initial internal and external ionic concentrations.

Similar considerations apply with regard to the changes in membrane resistance or the effects determined by the potassium-free solutions. It seems difficult to accept the idea that potassium will effect the physiological behavior of the membrane only through the ratio of its internal to external concentration. Its absolute concentration in the membrane, or the ratio of its concentration to the concentration of other ions, or even the ionic strength of the solution that forms the liquid phase of the membrane might be more important. These factors, and probably many others not known, may determine the state of the proteins (or conjugated proteins) that constitute the postulated membrane and through these mechanisms greatly affect the electrical activity of the cell.

The experiments in which the resistance of the membrane was measured with the electroplax kept in potassium-free saline, show that roughly half the conductance of the membrane at rest is due to potassium. It will be shown in a subsequent paper that chloride plays a small role in the membrane conductance and that about one-half of the current flowing across the membrane, regardless of the direction of flow, may be carried by sodium. These findings agree with the observations made with axons of *Carcinus maenas* (Keynes and Lewis, '51), and in striated muscle (Keynes, '54). However, the evaluation of the contribution of potassium to the conductance of the membrane, based upon experiments with potassium-free saline solution, may be erroneous. The efflux of sodium is markedly decreased in frog muscles immersed in potassium-free Ringer (Keynes, '54). Similar observations have been repeated with giant axons of *Sepia* (Hodgkin and Keynes, '55a). It appears difficult to evaluate, a priori, the rôle that actively transported sodium may have with regard to the conductance of the membrane, because it will depend upon the nature of the mechanisms of transport which are at present unknown. As in squid (Cole and Marmont, '42) and crab axons (Hodgkin, '47) the excess of potassium decreases the membrane resistance. The magnitude of this effect, however, varies widely from the results obtained by Hodgkin ('47) in crab nerves. In the latter case tripling the concentration of potassium decreased the membrane resistance by one-third. Furthermore, the decrease of resistance was proportional to the concentration of potassium at all the levels tested. In the case of the electroplax, a limiting value is apparently reached with a small increase of potassium.

The resistance of the membrane during the falling phase of the spike is surprisingly independent of the potassium concentration. The resistance during this phase is the same whether there is no potassium in the saline solution or whether its concentration is up to ten times the normal. At higher concentration (over 0.05 M) this resistance slightly diminishes but, as mentioned before, the membrane exposed to a high concentration of potassium probably may undergo such radical alterations that the information obtained may not be applicable to the normal membrane. Since the conductance of the membrane during the falling phase is practically independent of the potassium concentration, at least two possibilities must be considered: (I) the potassium conductance is practically nil during the falling phase of the spike, or (II) the membrane permeability is independent of the external concentration of potassium in this situation. The first hypothesis seems inadequate in the light of the experiments of Hodgkin and Huxley ('53) and Hodgkin and Keynes ('55b). These investigators demonstrated that the steady outward current associated with depolarization in giant axons is mainly due to the flow of potassium ions. It appears, therefore, that during this phase not only the ionic permittivity of the membrane decreases well beyond the magnitude at rest, but also some of its pharmacological properties are modified.

The conductivity of the membrane, increased during the rising phase of the action potential, does not merely return to the resting level during the falling phase. The total conductivity during this phase is actually about one-half to one-third of that at rest. The external concentration of potassium ceases to have any significant effect on this conductivity. This state may be reached without elicitation of an action potential, i.e. without the sudden net influx of sodium characteristic of the spike. This condition occurs when the activity of the electroplax is blocked by a high external concentration of potassium and the cell is sufficiently depolarized by an electric current. As stated above, the threshold depolarization for eliciting the spike in the normal cell and for determining the delayed increase in resistance in the electroplax exposed to an excess of potassium has the same magnitude. It may be suggested that in a normal cell the threshold depolarization triggers not only the process that will determine the increase in permeability of the membrane to sodium, but also the process or processes that will cause, after a suitable delay, the decrease of the conductivity characteristic of the falling phase. The experimental evidence available shows that the two series of processes may be interrelated only through the variations of the transmembrane potential produced by the influx of sodium. This is suggested by the fact that the rate at which the delayed phase of low permeability develops depends on the magnitude of the transmembrane potential, and is independent of the existence of a spike.

It might be added that this conception of a membrane changing rapidly to a series of successive states, each one with a characteristic ionic conductance, resembles closely the excellent formal description applicable to the squid axon made by Hodgkin and Huxley ('52b). The decreased permeability during the falling phase, apparently is related to what they called "inactivation process." A quantitative determination of the ionic flux in the electroplaque seems necessary before a profitable comparison with the squid axon may be offered. However, it must be stated that this is merely an attempt to describe the phenomena that occur during the action potential. Our knowledge of the biochemical changes taking place in the membrane is indeed extremely fragmentary. These changes must be known for a satisfactory understanding of the phenomena described.

ACKNOWLEDGMENT

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SUMMARY

1. The action on some electrical properties of the electroplax of saline solutions without potassium and with an excess of this ion has been studied.

2. The resting potential of the electroplax increases for at least one hour during the exposure to potassium-free solutions. Resting potentials of 140 mV or more have been measured.

3. The amplitude of the action potential increases by the same magnitude as the resting potential for about the first half hour of exposure to the potassium-free solution. Afterwards, the maximal rate of rise of the action potential diminishes and the spike may cease to be propagated. In this event, the overshoot is markedly reduced.

4. The resistance of the membrane at rest is doubled or tripled when potassium-free solution is used. It is concluded that potassium contributes to about half the conductance of the membrane at rest.

5. The resistance of the membrane during the falling phase of the spike is not modified by the lack of potassium in the saline solution.

6. The excess of potassium depolarizes the membrane to a magnitude that differs significantly from the values predicted by Henderson's equation.

7. Procaine in concentration sufficient to prevent the depolarization caused by acetylcholine and some of its derivatives or prostigmine does not modify the action of an excess of potassium.

8. Increase of potassium in the saline solution up to 0.025 M causes a decrease of the amplitude of the spike equal to the decrease of the resting potential and blocks the propaga-

tion of the former. A further addition of potassium (0.05 to 0.1 M) determines the abolition of the overshoot and, if the concentration is large enough or the time of exposure sufficiently prolonged, it causes the complete disappearance of all activity. The maximal rate of rise of the action potential is initially increased by an excess of potassium.

9. The increase of the potassium concentration in the saline produces a marked reduction (50% or more) of the resistance of the membrane at rest.

10. The resistance of the membrane during the falling phase of the spike is not modified by wide changes of the external concentration of potassium.

11. The membrane resistance reaches a magnitude equal to the resistance during the falling phase of the spike when exposed to a prolonged catelectrotonus, even after all activity has been blocked by an excess of potassium.

12. An anelectrotonus may markedly increase the amplitude of the activity previously inhibited by an excess of potassium (Woronzow phenomenon).

13. The findings enumerated above are discussed with regard to the physiological role of potassium in bioelectric potentials.

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DIFFERENTIATION OF THE EFFECTS OF pH AND CO_2 ON SPIRACULAR FUNCTION OF INSECTS

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ONE FIGURE

INTRODUCTION

While it is well known that high pCO_2 and low pO_2 induce opening of the insect spiracle (Hazelhoff, '26; Wigglesworth, '35), the mechanism and ultimate nature of the essential stimuli remain obscure. Concerning the opening stimulus associated with elevated pCO_2, the occurence in insects of the carbonic acid-bicarbonate buffering system (cf. Levenbook, '50) affords at least three possibilities. The stimulus may be (1) acidity, resulting from accumulation of acid metabolites of anaerobic metabolism as well as from H_2CO_3 derived from metabolic CO_2 (Wigglesworth, '35); or (2) dependent on more specific properties of CO_2; or (3) a combination of acidity and specific action of CO_2. To date, the only experimental evidence bearing on this question has been provided by Wigglesworth ('35) who concluded that responses of the flea, *Xenopsylla cheopis* Roths., to atmospheres from which both CO_2 and O_2 were being rapidly removed by alkaline pyrogallol, and to administration of lactic acid were indications that the essential stimulus to opening is acidity. This interpretation, however, is vulnerable to the argument that neither experimental procedure necessarily results in pH change independently of change in pCO_2. Obviously, a decisive analysis of stimulus specificity demands experiments in which these two factors are varied independently. The purpose of the present investigation has been to provide them.

MATERIALS AND METHODS

Laboratory reared female houseflies, *Musca domestica* L., and male and female fleshflies, *Sarcophaga bullata* (Parker), were used, the former one day and the latter between one and seven days after emergence. Except where specified, rearing techniques and methods of handling were as previously described (Case, '55).

Three types of experiments were made: vapor tests (i.e., exposure to air saturated with certain volatile compounds); small volume injections; and perfusions. The first two served to define the limits of response of intact flies. Since these experiments demonstrated that the critical factor for spiracle opening is either low pH or high pCO_2, perfusion experiments were undertaken to differentiate between them by eliminating blood buffer components to some degree while varying extracellular pH and CO_2 independently of each other.

All experiments were carried out with the insects in air at approximately 25°C. For vapor tests, perfusions, and most small volume injections, the flies were attached by their sides to pasteboard with Duco cement.[1] The left wing and left second and third legs were removed to facilitate observation of the left metathoracic spiracle, whose responses were judged and the resultant data analyzed according to Case ('55). Fleshflies were used only for certain small volume injections; female houseflies were the subjects of all other tests.

1. Vapor tests. Air saturated at 25°C. with acid and non-acidic irritant vapors was collected in 50 ml syringes and used to replace the air in 15 ml observation chambers containing from one to five flies.

2. Small volume injections. Solutions of acids and of their salts were prepared in distilled water, and, in the case of the houseflies, were injected into the antero-dorsal part of the thorax. A micrometer-driven 0.25 ml tuberculin syringe delivering through a glass needle with 0.25 mm outside tip diameter was used for injection. The same apparatus was used to deliver solution into regions near a given spiracle in fleshflies.

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[1] E. I. DuPont Nemours and Co., Wilmington, Delaware.

3. *Perfusions.* Houseflies were perfused with approximately 0.4 ml of various buffered solutions at a rate of approximately 0.4 ml/min. Fluid entered the anterodorsal region of the thorax through a 27 ga. hypodermic needle connected with a micrometer-driven 1.0 ml tuberculin syringe. An exit was provided by an abdominal incision into which was inserted a filter paper wick. The perfusion fluids were formulated so that pH, [CO_2], and total buffer concentration might be varied (table 1). All perfusion solutions were prepared on the day of use and stored under a 1 cm layer of mineral oil in glass stoppered bottles. Determinations of pH of the perfusion solutions were made before and after each experiment with a Beckman Model G pH meter. The pH standard was Fisher Scientific Company Buffer Solution, pH 7.0 ± 0.01 at 25°C.

TABLE 1

Composition of perfusion solutions

TEST DESIGNATION	A	B	C	D
Variable Constant	pH[1] [CO_2]	pH[2] [CO_2]	pH[2] [CO_2]	pH[2] [CO_2]
NaCl	7.50[3]	7.50	3.36–6.82[4]	Same as B except no H_3BO_3
KCl	0.35	0.35	0.35	
$CaCl_2$	0.21	0.21	0.21	
H_3BO_3	1.30	1.30	. . .	
NaH_2PO_4 H_2O	0.14	. . .	. . .	
$NaHCO_3$	. . .	1.76	1.00–6.00	

[1] pH adjusted with HCl and NaOH.

[2] pH adjusted with CO_2.

[3] Grams/liter distilled H_2O.

[4] For a given concentration of $NaHCO_3$, NaCl concentration was adjusted to give M Na^+ equivalent to A.

RESULTS

1. *Vapor tests and small volume injections.* These experiments confirm and extend the observations of Wiggles-

worth ('35) to the effect that lactic acid may induce spiracle opening.

The resultant data, table 2 (A), also indicate a striking difference in response of the spiracular apparatus, depending on whether the organs of taste and olfaction are intact. The normal fly evidently responds to a great many volatile compounds with violent avoidance reactions. Opening of the

TABLE 2

Test of specificity of spiracular responses in houseflies

(In terms of per cent response scores)

SENSES OF TASTE AND OLFACTION		INTACT		ELIMINATED	
Compound tested		Score	N	Score	N
(A) Vapor tests (air saturated with the given compound at 28°C.)					
Acids:					
Acetic		100	20	100	20
Butyric		100	20	85	20
HCl		100	20	95	20
Alcohols:					
Methyl		85	20	0	20
Ethyl		75	20	0	20
Iso-propyl		95	20	0	20
Miscellaneous:					
Toluene		85	20	0	20
Acetone		100	20	0	20
Chloroform		95	20	0	20
(B) Small volume injection tests (injection volume is 2.0 mm^3 except where specified)					
Lactic acid	0.125 M	100	20		
Na lactate	.125 M	0	20		
Citric acid	.125 M	90	20		
Na citrate	.125 M	0	20		
NH_4Cl	.300 M	0	12		
	.600 M	55	20		
NaCl	.600 M	12	16		
H_2O	2.0 mm^3	0	10		
	3.0	0	10		
	4.0	50	10		

spiracles invariably follows after a slight interval, an indication that opening is probably in response to the sequelae of muscular activity rather than a direct effect of the test compound. After the organs of taste and olfaction have been removed or covered with paraffin, these violent movements ordinarily do not occur; and under these conditions spiracular opening appears to be stimulated only by CO_2 and acids.

None of the substances tested as vapors in this manner permanently impaired spiracle function. Spiracles always closed subsequent to the test and were for several hours thereafter responsive to changes in pCO_2 and pO_2.

Small volume injections provide a similar picture, table 2 (B). Lactic and citric acids (0.125 M) caused immediate and reversible spiracle opening on injection of 2.0 mm^3 into houseflies. NH_4Cl was also somewhat effective, suggesting that the response is evoked by acidity in general and not only by acidic metabolic intermediates.

The large size of the fleshfly made possible observation of the effects of localized injections of lactic acid. Injection of 2 mm^2 of 0.125 M lactic acid within 0.25 mm of a given thoracic spiracle is followed by immediate opening of the spiracle, after which transitory opening of the other thoracic spiracles occurs at intervals varying from 15 seconds to one minute. While the relatively long interval between opening of successive spiracles may indicate nothing more than diffusion and circulatory transport of the lactic acid, neural involvement cannot be eliminated from consideration. These matters will be considered further in a subsequent publication.

Injection of 2 mm^3 of 0.075 M lactic acid usually caused opening of only the near spiracle while 0.050 M was seldom effective.

2. Perfusions. Perfusion of houseflies with at least 1.0 ml of solution B (cf. table 1) at pH 7.0 produces no overt harmful effects within four or more hours.

The first series of perfusions (table 3) was designed to compare the effects on the spiracles of changing $[H^+]$ without change in $[CO_2]$ by means of perfusions with solutions of type A; and of simultaneously varying [H^+] and [CO_2] by means of perfusions with solutions of type B. Employing as the criterion of response the state of the metathoracic spiracle at the instant of termination of perfusion, there could be little doubt that acidity, in the absence of increased [CO_2] of the saline, is completely ineffectual within a reasonable physiological range. Solution A at a pH at least as low as 4.2 *never* caused spiracle opening. On the contrary, when this solution was used to perfuse an insect which had spiracles open as a result of muscular activity, closure regularly occurred even though muscular activity might continue.

TABLE 3

The effects of low pH alone and of low pH together with high pCO_2 on (in terms of percent response scores)

VARIABLE COMPONENTS	pH 4.2	4.5	6.0	6.25	6.5	6.8
pH (Solns. A, table 1)	0 (25)[1]	0 (15)	0 (15)	0 (15)	..	..
pH and pCO_2 (Solns. B, table 1)	..	..	100 (15)	60 (15)	40 (15)	10 (15)

[1] Numbers in parentheses indicate number of flies tested.

There seems little reason to suspect that spiracle closure at this very low pH is due to a direct stimulation of the spiracle muscle by acidity since direction of a stream of CO_2 at the spiracle of the perfused insect causes opening, that is, relaxation of the spiracular valves. It will be recalled that the thoracic spiracular valves of the housefly are each operated by a single occlusor muscle, opposed by the elasticity of the tracheal wall (Hassan, '44).

The effectiveness of solutions of type B, in which both [H^+] and [CO_2] are varied, is in striking contrast with the results just described for solutions of type A in which [CO_2] is uniformly very low. Perfusion with type B solutions produces a 50% spiracular response between pH 6.25 and pH 6.50

(table 3) or, in terms of molarity of H_2CO_3, 0.015 and 0.008 M. Clearly, the effectiveness seen here must be associated with CO_2 since both solutions A and B were tested in overlapping pH ranges and had similar compositions except for CO_2 and sodium acid phosphate.

Perfusion of *Drosophila gibberosa* Patterson and Mainland has also shown buffer type B to be effective at pH 6.0 while type A is without effect on the spiracle.

The second perfusion series, solutions of type C *vs.* D, confirms the concept of the efficacy of CO_2 in another fashion. In this case, the spiracular response was evaluated on exposure to perfusion fluids of variable $[H^+]$ and $[CO_2]$ (type D) and to fluids of constant $[H^+]$ and variable $[CO_2]$ (type C). With the type D fluids, the bicarbonate concentration was held constant so that $[CO_2]$ varied with pH, as with type B. In the case of type C solutions, pH was kept constant at 6.5 as $[CO_2]$ was varied by proportional variation in both $[CO_2]$ and $[NaHCO_3]$, according to the Henderson-Hasselbach relationship.

If the characteristic of the perfusion fluid which is essential to spiracle opening is $[CO_2]$ and not pH, then solutions C and D ought to be equally effective when compared in terms of H_2CO_3. This actually is true, as may be seen from figure 1, where spiracular responses to both perfusion fluids are plotted in terms of molarity of H_2CO_3. Both solutions have very nearly identical ED_{50}'s, approximately 0.011 M H_2CO_3, and the slopes of the dose response curves are statistically indistinguishable. Again, it must be concluded that the actual pH of the perfusion fluid is unimportant and the pCO_2 remains the governing factor.

DISCUSSION

At least one major difficulty is attached to the interpretation of these observations. While it has been adequately demonstrated that CO_2 in the extracellular environment is considerably more significant to spiracular function than is pH change alone, we are left with no concept of the relative importance of pH and CO_2 within the receptor cell itself. Should the site at which the spiracular response is initiated lie separated from the general circulation by a cytoplasmic

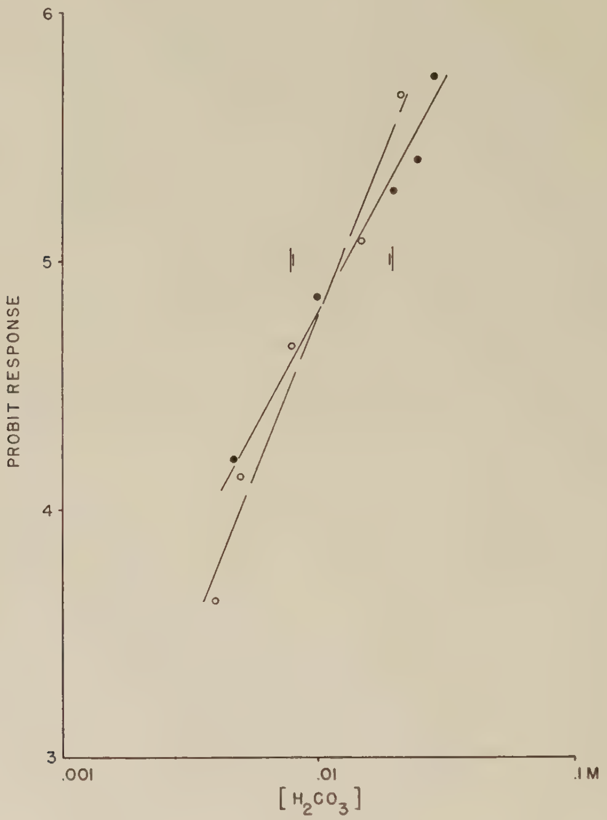

Figure 1

Dose-response lines for effects of two types of perfusion fluids on spiracular opening in the female housefly. Ordinate: Probit of per cent spiracular opening response, i.e., 5 = 50% response score. Abscissa: molarity of H_2CO_3.

	PERFUSION TYPE	[CO_2]	pH	ED_{50} ± S.E. (M H_2CO_3)	Slope ± S.E.	N
Solid line, closed points	C	variable	6.1–6.8	.0109 ± .0021	2.514 ± .343	209
Broken line, open points	D	variable	6.5	.0107 ± .0019	1.864 ± .303	261

membrane, there exists the possibility that pH change at this site is still the governing mechanism. The greater effect of CO_2 in the extracellular medium would in this case be attributed to the greater ease, as compared with H^+, with which it penetrates the membrane of the receptor cell. While the receptor cell, as well as the properties of its membrane, are unknown, there is evidence that CO_2 tends to penetrate cell membranes more rapidly than H^+ (for examples, see Brooks and Brooks, '41). Gesell ('39) has, in fact, proposed such a mechanism for control of the mammalian medullary respiratory center, i.e., penetration of CO_2 into the receptor cell, causing H^+ release. Consequently, there appear to be sound reasons for limiting the interpretation of the present experiments strictly to the extracellular environment.

The high degree of effectiveness of acids in gaseous form and of injections of small volumes of acids in inducing spiracle opening may therefore best find explanation for the present in liberation of CO_2 from buffer components of blood and tissues. The experiments just reported have shown that low pH will induce spiracle opening only in the presence of normal amounts of hemolymph. When perfusions are employed, in volumes which certainly must vastly reduce the quantity of hemolymph, CO_2 appears to be the only effectual agent. Since the hemolymph of insects contains combined CO_2 which may be released by acid (Levenbook, '50) the most reasonable conclusion seems to be that acid is effective in spiracle opening *via* the release of bound CO_2.

The same interpretation is applicable to the two experiments advanced in support of H^+ activation of the spiracular mechanism by Wigglesworth ('35). His experiment in which spiracular response occurred when the cut thoracic surface of a decapitated flea was bathed in lactic acid does not prove the primary role of acidity since CO_2 release from hemolymph buffer components was not ruled out. Wigglesworth's second experiment, in which a flea was confined in a small chamber from which CO_2 and O_2 were continuously extracted by alkaline pyrogallol might equally well be reinterpreted. First, the effects of the hypoxia must be considered. Although low O_2 renders the spiracular apparatus more sensitive to CO_2 (Case, '54, '55), the mechanism is not fully understood. O_2 effects may be of a primary sensory nature, may consist of generalized metabolic effects, or, finally, may involve a combination of both. In the first instance O_2 lack is envisaged as causing spiracle opening through the activity of specific O_2 receptor elements of the nervous system. In the second, a metabolic consequence of hypoxia, such as lactic acid production, would be perceived by a CO_2 receptor via CO_2 release in the tissues, since acid accumulated during anoxia is known to release CO_2 from bicarbonate in insects as well as in other animals (Harnisch, '30; Slater, '28). Secondly, whichever oxygen effect or combination of effects is involved, the experiment with hypoxic fleas suffers in precision of interpretation by the undoubted presence of acid-labile CO_2 in the hemolymph. Although CO_2 was removed from the environs of the flea, the critical factor, rate of removal from the flea, is unknown. The general impermeability of the insect cuticle to gases (Fraenkel and Herford, '38; Ito, '53), with the consequent necessity of removing CO_2 largely by way of the limited spiracular pathway, creates the possibility that higher than normal CO_2 tensions might be attained early in the experiment. This coupled with the CO_2 sensitizing effect of the increasing hypoxia, could explain the initial spiracle opening. By the time all available CO_2 had been extracted from the insect, the advanced state of anoxia, *per se,* might be sufficient to maintain the spiracle occlusor in the relaxed state (Case, '54). Viewing all the experimental evidence in this light, we appear to be left with no data damaging to the concept of the primary importance of CO_2 in the extracellular environment as the initiator of spiracle opening.

SUMMARY

Experiments on houseflies, *Musca domestica* L., and fleshflies, *Sarcophaga bullata* Parker, have shown that only acids and CO_2 cause spiracle opening in insects deprived of olfac-

tory and gustatory senses. The primary importance of CO_2, rather than acidity, to the opening reaction is shown by perfusion experiments in which pH and $[CO_2]$ were varied independently.

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CHARACTERISTICS OF THE DIURNAL RHYTHM OF LUMINESCENCE IN GONYAULAX POLYEDRA[1,2,3]

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SIX FIGURES

INTRODUCTION

The production of light by living organisms is to a great extent associated with the marine environment (see Harvey, '52). *Gonyaulax* is a marine dinoflagellate which is both photosynthetic and luminescent, being one of the many organisms which contribute to the frequently observed "phosphorescence" of the ocean. Its luminescence occurs upon stimulation, as a bright flash having a duration of about 0.1 sec. When large numbers of these organisms are stimulated in the ocean, as by the passage of a school of fish or by wave action, the disturbed water glows as a result of their flashing. Fishermen locate sardine schools by such luminescence.

Dinoflagellates in general have been reported to lose their ability to luminesce in daylight (see Harvey, '52). Such a phenomenon is also known in other luminous forms, particularly the ctenophores. In the latter, the ability to luminesce

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[2] We are grateful to Mr. William K. Purves for his assistance in carrying out these experiments.

[3] Contribution from the Scripps Institution of Oceanography, University of California, New Series No. 911.

is rapidly inhibited in light, but recovers after a sojourn of 20 to 30 minutes in darkness. In the case of dinoflagellates, however, reports by several observers (Zacharias, '05; Moore, '08; Kofoid and Swezy, '21; Harvey, '52) suggest that luminescence is under the control of a type of diurnal rhythmic mechanism. This conclusion is based mainly on visual observations that dinoflagellates kept in total darkness fail to luminesce during the day, but regain their ability to do so during the nighttime. The studies described in this paper confirm this general conclusion with quantitative measurements, and give additional information concerning other effects of light.

MATERIALS AND METHODS

A unialgal culture of *G. polyedra* was isolated by one of the authors (B. M. S.) from a net sample collected off the pier at Scripps Institution of Oceanography, on September 22, 1952. Since that time, the cultures have been maintained in Fernbach flasks placed in a window (north exposure, 400–800 foot candles) at room temperature (23°–26°C.). The culture medium was composed of 75% filtered sea water, 2% soil extract (Pringsheim, '46), 2×10^{-3} M KNO_3, 2×10^{-4} M K_2HPO_4, 6×10^{-6} M $FeCl_3$, and 2.7×10^{-5} M ethylendiaminetetraacetic acid (EDTA) at pH 8.0–8.2. All distilled water used in these experiments was distilled a second time in a Pyrex glass still.

For experimental purposes, some cultures were grown and maintained in a temperature-controlled cabinet (25°C.) under constant illumination at an intensity of about 700 foot candles. Illumination was provided by "cool white" fluorescent lamps. Cultures grown in the window were used for experiments in which it was desired that organisms be exposed to normal day-night changes in light intensity. Cultures were inoculated at a cell density of about 800 cells per milliliter, and were used for experiments 10–15 days later at a population density of 6000–13000 cells per milliliter.

The luminescence of *Gonyaulax* may be induced by mechanical stimulation. After a few seconds of agitation, the luminescent response is markedly decreased. Agitation during the pipetting of population samples is unavoidable. Although recovery was not studied in any detail, it was determined that a rest period of two hours in the light is sufficiently long to permit recovery from agitation coincidental to the preparation of samples. For experimental studies, 2 ml aliquots from cultures were pipetted into groups of test tubes and given a recovery period of at least six hours in the light. Luminescence was assayed by stimulating the cells in test tubes with a fine jet of purified air from a compressed air tank. Since it was impossible to determine luminescence accurately from the intensity recordings, the output from the phototube was accumulated in a capacitor, giving a measure of the total light emission (see fig. 1). This method was used for all assays.

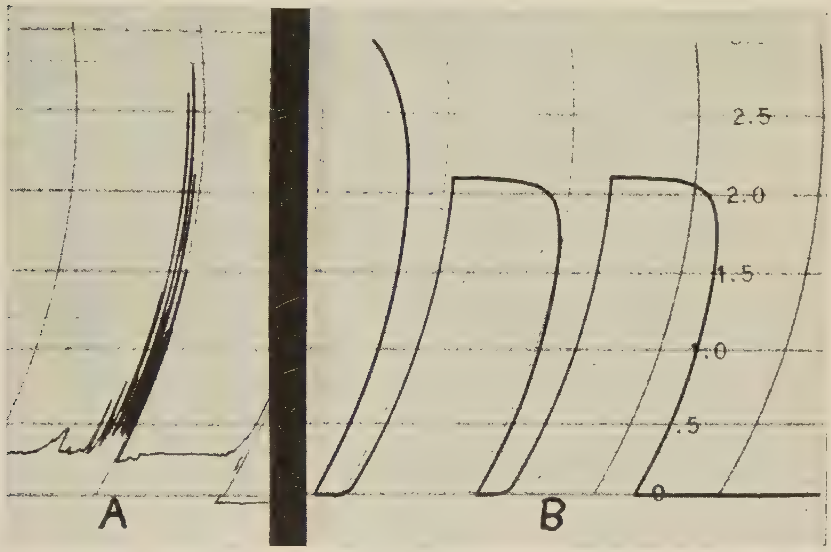

Fig. 1 Recordings of *Gonyaulax* luminescence. A. Intensity measurement. B. Measurement by integration. Time scale, one minute between vertical divisions. Intensity measurement does not give an accurate record of flashing since the flashes have a duration of 0.1 sec. and the time constant of the meter was 0.5 sec. Direction of time axis is from right to left.

Since essentially all luminescence was exhausted within one minute, the values for luminescence of cells are expressed in terms of total (integrated) light emitted during one minute of stimulation, expressed as arbitrary total light units. With the exception of this integration circuit, the light-measuring apparatus was essentially the same as described previously (Hastings, McElroy and Coulombre, '53), utilizing a 931A photomultiplier tube and an Esterline-Angus graphic meter for recording. Provision for precision voltage regulation makes the sensitivity of the instrument independent of time of day.

After each determination, the experimental tube was discarded. Determinations made from a number of aliquots from a given culture under similar conditions generally agreed within 10%, but very occasionally greater variations were encountered. These cases may have been due to any of a variety of factors, and we were not successful in eliminating them. Individual experimental determinations which varied widely from others, although included in the figures, are therefore not considered to be highly significant.

Time of day is expressed throughout in Pacific Daylight Time, and the hours are numbered consecutively from 0100 (1 A.M.) to 2400 (midnight).

EXPERIMENTAL RESULTS

Haxo and Sweeney ('55) have shown that cells grown in bright continuous illumination have a dim luminescence which shows no detectable rhythmic variation. Using cells grown under these conditions, they found that changes in luminescence could be initiated by placing the cells in total darkness. Cells kept in darkness over a period of 40 hours were observed to increase in brightness during the first 6–8 hours followed by a decline. A second increase in luminescence followed this with a maximum between 20 and 24 hours after the first peak.

We have repeated these experiments with cells which had been cultured in constant light for 8 weeks and extended the period of darkness up to 100 hours. The results of such an experiment are shown in figure 2. The fluctuations suggest that *Gonyaulax* cells possess a type of "biological clock," or an inherent mechanism of rhythmicity which is inhibited in continuous illumination but which may be initiated at any time by subjecting them to darkness. The period between peaks has been found to be about 23 hours in these experiments. The magnitude of each succeeding peak is progressively lower, suggesting the exhaustion of the energy stores. Since energy in this organism is derived from light through photosynthesis, this is an expected consequence of continuous darkness. The fall in peak height with time is

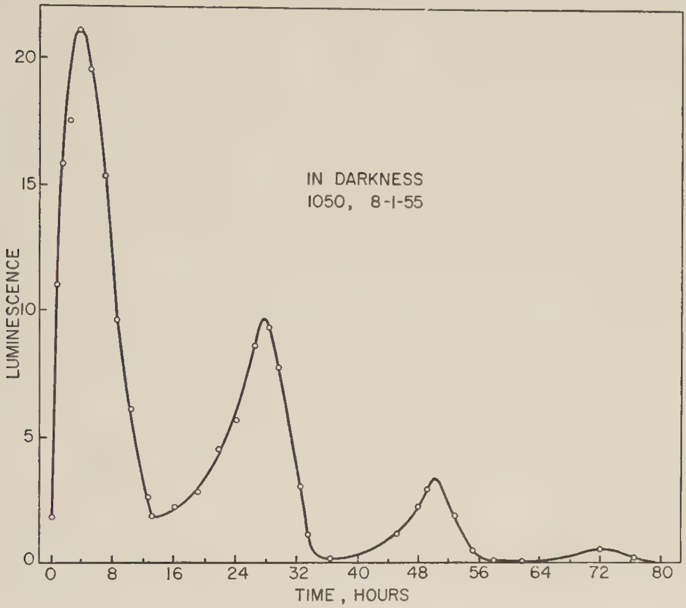

Fig. 2 Spontaneous rhythmic changes in luminescence of cells grown in constant light and placed in total darkness. Luminescence in constant light, 1.8 light units. Time in hours after placing in darkness. Each point represents the average of 2–4 determinations.

slightly greater than logarithmic over the long continued dark period, while cell counts decrease only slightly, and there is no detectible decrease in the per cent of cells which are motile. Consequently, the progressive reduction in the magnitude of successive peaks in luminescence probably reflects a progressive starvation of all the cells of the population, rather than a reduction in the per cent viable cells.

It was of interest to re-examine whether or not cells retained any detectible rhythmicity while in constant illumination. This was done in two ways. We first made additional measurements to determine whether or not there were any significant fluctuations in the dim luminescence of cells kept in constant light. No variations which indicated a rhythm were observed. Secondly, an experiment was planned to test whether or not the initiation of the rhythm in cells grown in constant light was independent of time of day. Aliquots of cells which had been grown in continuous light were removed to total darkness at three hour intervals over a 24 hour period. The first part of the rhythmic luminescent cycle of each group was determined. The results of this experiment are summarized in figure 3, and show that in light-grown cultures, the rhythm may be initiated with equal rapidity at any time of day. A systematic variation in the maximum luminescence attained was observed, the greatest luminescence being developed between 1300 and 1500. An experiment carried out over a longer time period is necessary to determine whether or not this variation represents a vestigial rhythmic pattern, displaced in time. However, it is apparent that the time intervals of the newly-developed rhythmic pattern are not affected.

It may also be noticed in the experiments shown in figures 2 and 3 that there is a considerable difference in the ratio of luminescent intensity in constant light to the maximum intensity in darkness. Wide variations in this ratio were encountered from culture to culture. Since aliquots from any one culture at any given time behaved uniformly, this variation appears to be caused by differences in the pretreatment of cultures. It was not possible to standardize pretreatment with precision with the equipment available and a growth medium which is not completely defined.

The changes in the luminescence of *Gonyaulax* exposed to natural day-night variations in light intensity was next studied, and the results are shown in figure 4. For this purpose, samples were pipetted from window-grown cultures and placed in the window. They were then assayed for luminescence at the times indicated. Figure 6 shows a more detailed measurement of a nighttime excursion.

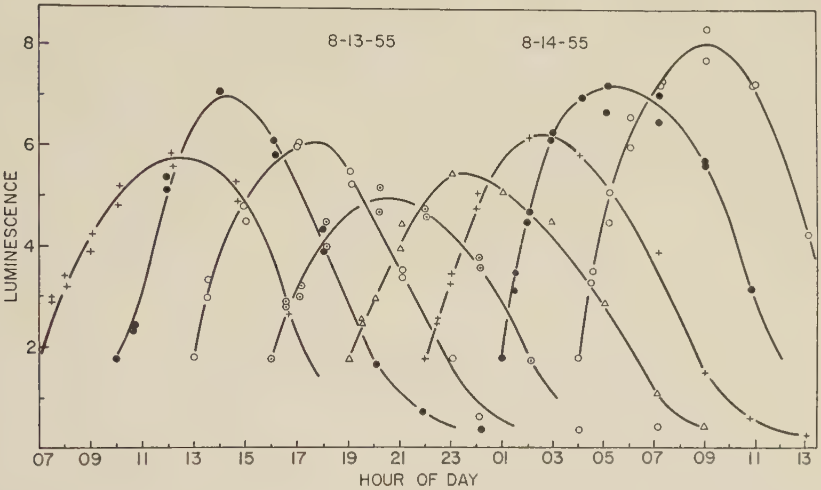

Fig. 3 Plot of the first cycle of luminescence (as in fig. 2, first 12 hours) in a series of experiments in which cells grown in constant light were placed in darkness at 3 hour intervals over a 24 hour period. Time (PDT) from 0700 hours (7 A.M.) of first day through 1300 hours (1 P.M.) of second day. The first determination of each curve was made at the time the aliquots of this group were darkened. Each point represents a single determination.

It can be noted (see fig. 6) that the luminescence begins to increase only at the onset of darkness, but begins to decrease before any light of morning has appeared. This suggests that the same mechanism is functional in the early morning decline of luminescence as in the decline observed in continuous darkness (fig. 2). To test this assumption, a number of aliquots from the window were removed during darkness (at midnight) and placed in total darkness. Their luminescence was assayed and found to decrease from the nighttime peak in the same manner as those left in the window (see fig. 4). Moreover, although the cells were kept in darkness all the while, their luminescence increased the following night.

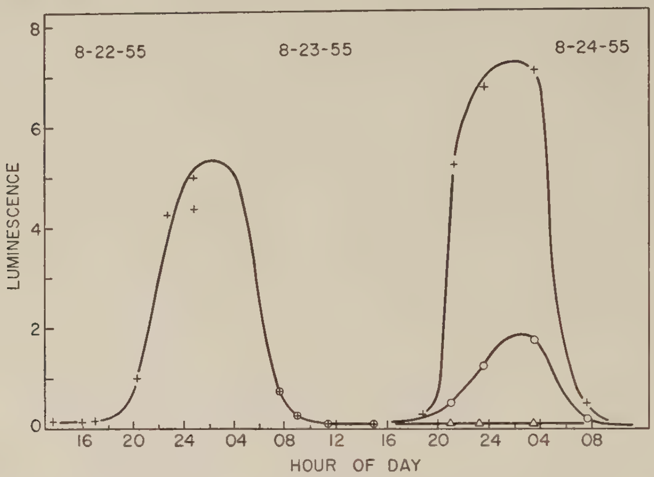

Fig. 4 Variation in luminescence over the course of two days of cultures grown and kept in window (indicated by crosses). Some cultures were removed from the window at midnight on the first day and put in total darkness (circles). Other cultures were removed from the window at noon and placed in constant light (triangles). Hour of the day plotted as in figure 3. Each point represents 1–2 determinations.

They differed from those left in the window in that the increase in luminescence the second night was not as great as that of those which were left in the window, and it occurred somewhat earlier. This latter point is more clearly demonstrated in a similar experiment shown in figure 5.

With the exception of the two effects noted above, the rhythm of the luminescence of *Gonyaulax* in the window continues unaltered when the cells are placed in total darkness at any time of the day or night. This is apparent from figure 5, where aliquots from the window were placed in continuous darkness at three different times during daylight. As can be seen, there was no increase in the luminescence in any of the experimental tubes until early evening, about one hour before the controls from the window began to increase in intensity. It may be possible that this earlier rise is related to the 23-hour period observed in cultures placed in continuous darkness (fig. 2). Moreover, the magnitude of the evening peak is related to the duration of the exposure to daylight, suggesting that "day length" (i.e. time × intensity) is effectively the important factor with regard to the magnitude of the nighttime peak. It seems probable that restoration of energy stores through photosynthesis is responsible for this light effect although direct evidence to support this assumption is not yet available.

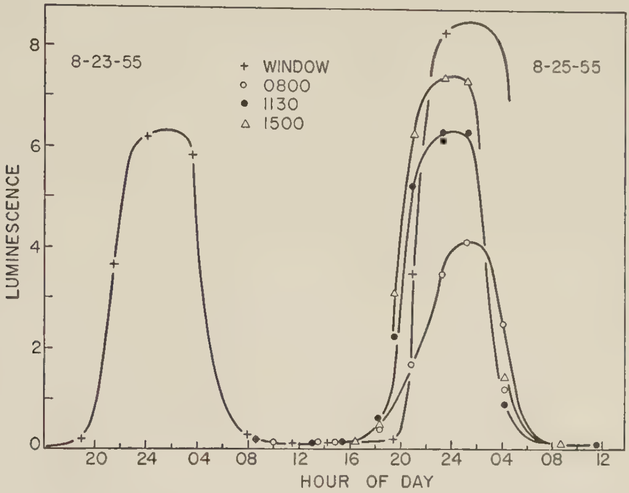

Fig. 5 Variation in luminescence over two days as in figure 4 (crosses). During the day three groups were removed from the window at the times indicated and placed in total darkness. The luminescence of each group was assayed at the times indicated, showing the effect of light during the day upon the amount of luminescence at night. Hour of the day plotted as in figure 3. Each point represents a single determination.

Another series of experiments was concerned with the effect of removing cells from the day-night regime in the window to the illuminated incubator. When cells were removed from the window to continuous light at any time during daylight, no subsequent increase in luminescence was observed (fig. 4). But on the other hand, if the cells were removed from the window and placed in constant light during the nighttime period (i.e., at any time after an increase in luminescence had already occurred) luminescence was decreased in all cases (fig. 6), as previously described by Haxo and Sweeney ('55) for cells exhibiting maximum luminescence. However, in no case is the luminescence decreased to the low day-time level, even when measurements were extended to 1100 the following morning. Thus, although light will inhibit any increase in luminescence when illumination is begun during daylight, once this increased luminescence has begun to develop in darkness, then inhibition by light is only partial. Thus light during the

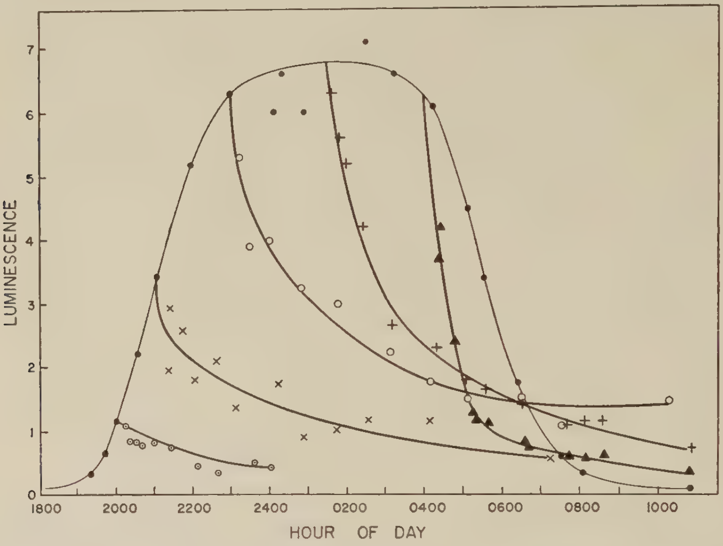

Fig. 6 Effect of light during the night upon the luminescence of cultures growing in the window. At 2000, 2107, 2300, 0130 and 0400 cultures were removed from the window and placed in constant light in the incubator, and the luminescence of these along with the control in the window was determined at the times shown. Time of day plotted as in figure 3. Each point represents a single determination.

night prevents the maximum excursion of the luminescent cycle, both up-swing and down-swing.

DISCUSSION

From the experiments described, it is clear that the luminnescence in *Gonyaulax* cannot be maintained at maximum brightness under any of the experimental conditions used. A maximum is always followed by a fall in luminescence. This alteration of bright and dim periods is observed in continuous darkness as well as during the normal succession of day and night, and so may be considered an endogenous diurnal rhythm, similar in nature to rhythms which have been described for a variety of activities in numerous organisms (see Bünning, '52; Kleitman, '49; Grossenbacher, '39; Welsh, '38). Since *Gonyaulax* is a unicellular form, the origin of the rhythmicity is evidently intracellular.

Under normal conditions of daily changes in light intensity luminescence is maximum during darkness and minimum during daylight. It has been possible recently (Hastings and Sweeney, '57) to demonstrate luminescence in cell-free extracts and to separate substrate and enzyme (luciferin and luciferase) from one another. It was found that extracts prepared from cells during nighttime were from 5 to 15 times more active than those prepared during daylight. At least part of the diurnal fluctuation in light intenstity may therefore be attributed to actual changes in the concentrations of luciferin and luciferase. It has been shown by Buck ('37) that spontaneous firefly flashing involves a diurnal rhythm. In the case of the firefly, however, the rhythm is evidently linked to the control of flashing and not to the production of photogenic substance. We have made some observations which suggest that *Gonyaulax* cells also may be inherently more susceptible to stimulation during the nighttime period than during the day. Cells from the window placed in a test tube in total darkness but not stimulated show a marked increase in the frequency as well as magnitude of what appears to be spontaneous flashing during the nighttime period. This may account for the fact that the difference in the luminescence of intact cells between day and night is greater than the corresponding difference in the activity of extracts by day and night. It is thus possible that both factors are involved in the changes in luminescence, and that both are linked to a rhythmic mechanism.

The shifting of the rhythm has not been studied in any detail. The results obtained thus far suggest that it may be shifted at will with the proper manipulation of light and dark periods. Certainly after long exposure to continuous light it may be initiated at will. This condition is similar to that found by Buck ('37) for spontaneous firefly flashing.

It was not possible to determine how long the rhythm itself might persist, since the cells apparently die within about 100 hours in continuous darkness. The fact that cells in constant light retain the mechanism over a long period of time suggest, but does not prove, that the mechanism is a persistent one. This point might be settled if a substance supplied in the medium could be utilized by the organism as an energy source. A number of compounds have been tried in this connection, but none have substituted for light insofar as growth is concerned (W. H. Thomas, '55). It is also possible, as indicated in preliminary experiments of Haxo and Sweeney ('55) that the blue-violet portion of the spectrum is most effective in inhibiting luminescence. In this case red light might be used to keep the cells alive while not disturbing the rhythms of luminescence.

Light may evidently affect the cells in a number of different ways. First it provides an energy source for the organism through photosynthesis. Luciferin and luciferase would be expected to be synthesized and destroyed daily via reactions which require energy. A decreased exposure to light with consequent reduction in photosynthesis would therefore result in a lower concentration of the photogenic compounds. Secondly, light will apparently inactivate or inhibit the luminescent system or its components. At the light intensities used, the inactivation is never complete. This may be a consequence of a balance between a photoinactivation and biosynthesis. Alternatively, the decrease in luminescence may be the result of the photoproduction of an inhibitor which, in the concentrations produced, is not 100% effective. Finally, as an independent effect, light appears to inhibit rhythmic changes in the intensity of luminescence.

A clarification of these various effects of light, and to what extent they are independent of one another, will be greatly aided by a determination of their respective action spectra. Studies of this nature are being carried out at the present time.

SUMMARY

The luminescence of cultures of the marine dinoflagellate, *Gonyaulax polyedra,* receiving light from the window, is dim during the day and about sixty times brighter at night. These variations may be described as a type of inherent diurnal rhythmicity, since a number of experiments demonstrate that the periodicity is maintained in the absence of normal day-night variations in light intensity. If the cultures are transferred from the window to total darkness at any time, the rhythmic changes in luminescence continue to occur, approximately in phase with cells left in the window. Continuous exposure to light will inhibit the rhythm, but it may be initiated in such light-treated cultures at any time by placing them in total darkness. The rhythm will then persist for about four days. The amount of luminescence which develops at night is directly related to the amount of light received during the day. This is probably related to the energy stores derived from photosynthesis. Light will also directly inhibit luminescence at any time during the night. The changes in luminescence are at least partially due to synthesis and destruction of luciferin and luciferase. It is also possible that the cells are more susceptible to stimulation during the night phase.

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ON THE ROLE OF HYDROGEN ION AND POTASSIUM ION IN THE ACTIVE TRANSPORT OF SODIUM ACROSS THE ISOLATED FROG SKIN [1]

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NINE FIGURES

This paper deals with (1) the production of a hydrogen ion gradient across the isolated frog skin, (2) the relationship between the gradient and the active transport of sodium ion, and (3) the effect of potassium ion and aearation on the hydrogen ion gradient and the transport process.

It has been known for many years that the isolated frog skin is able to change the pH of the bathing solutions (NiiNa, '23; Gicklhorn, '33; Steinbach, '37; Ussing, '49a; Huf and Wills, '51, '53; Levinsky and Sawyer, '53) but the significance of such changes is not known. Meyer and Bernfeld ('46) found that if the epidermal side of the skin was bathed in tap water, and the corium side with phosphate buffers of various pH, the changes of potential observed closely followed the behavior of a glass electrode, i.e., a change of about 55 mv. for each pH unit. They suggested that the potential arose because the skin contained a membrane which was selectively permeable to hydrogen ion.

Ussing ('48, '49a) made use of the findings of Meyer and Bernfeld ('46), suggesting that the active transport of sodium

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ion might, in fact, mean a forced exchange of sodium ion for hydrogen ion, the rate of movement being that which would keep the intracellular pH constant. Huf and Wills ('53), noting the inequality of sodium and chloride movement, suggested that not all of the sodium ion appearing in the solution bathing the corium was transported, but rather that some sodium might be exchanged for hydrogen ion produced by the metabolic activities of the skin. From their studies it appeared that the chloride movement might better express the net amount of sodium pumped.

In view of the foregoing suggestions, it seemed worthwhile to study the production of hydrogen ion by the isolated skin. By using a volume-area ratio close to one, and unbuffered solutions, the suggestions of Ussing ('48, '49a) and Huf and Wills ('53) could easily be checked, measuring both hydrogen ion production and sodium transport as a function of time. Under such conditions, the pH of the solution bathing the epidermal side of the skin should fall as sodium is being either transported, or exchanged for hydrogen ion, across the skin.

EXPERIMENTAL

The studies reported here were carried out using plexiglass chambers of the type shown in figure 1. The skin serves as a membrane separating the two half-cells which are machined to fit closely together. In practice, a frog is killed by decapitation, the spinal cord destroyed, and the abdominal skin removed. A circular disk of skin which just fits the cells is cut, using a special knife constructed in the manner of a cookie cutter, and mounted in the female half of the cell. The skin and two half-cells are held together with gentle pressure from a suitable C clamp. The area of skin exposed is 7.1 cm^2, and usually 7.1 ml of solution is used on each side. Mixing and aeration are accomplished by means of air lines which open into the bottom of each half-cell. Potential differences are followed by means of calomel electrodes which dip into KCl wells, which in turn connect with agar bridges which open into the chamber at a distance of 0.5 mm from the surfaces of the skin. Changes in pH are followed by inserting glass electrodes into the openings at the top of each half-cell.

The chamber has the advantage that small changes in the parameters measured may be easily followed. It has the disadvantage that errors due to evaporation may be appreciable if not corrected. It was found that the air supply should be passed through at least two gas washing bottles if evaporation was to be kept at a minimum. By replacing the frog skin with a teflon disk, and measuring the increase in the concentration of Na^{22}, it was found that the water loss amounts to about 0.01 ml/hr under such conditions.

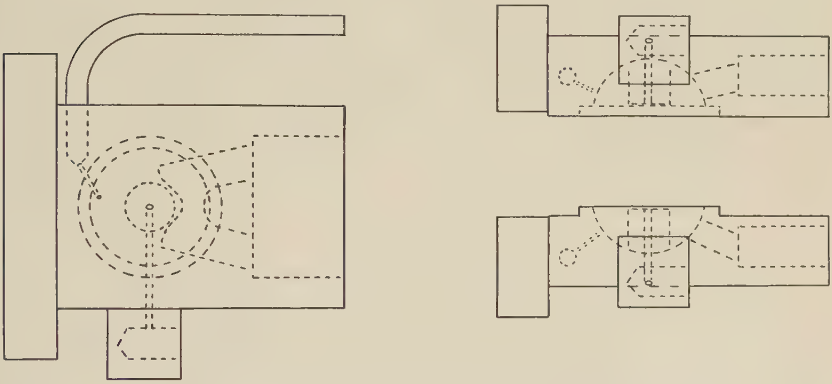

Fig. 1 Drawing of the plexiglass cell used to follow sodium transport, potential differences, and pH changes across the isolated frog skin.

Sodium analyses were carried out using a Beckman flame photometer, and later, Na^{22}. The method used is indicated in the data. The sodium isotope was obtained from commercial sources, and sufficient Na^{22} was added to give at least 100 c.p.m. for each microequivalent of sodium used. Aliquots (0.05 ml) were pipetted into counting plates containing a disk of filter paper, a drop of glucose solution was added, and the solutions were evaporated to dryness before counting. The experiments cited with the flame photometer were carried out in the spring and summer months of 1953 and 1954. The Na^{22} experiments were carried out in late November and December of 1955, and January of 1956.

When the flame photometer was used, the occurrence of active sodium transport was indicated if (1) sodium ion accumulated in the solution bathing the corium, and (2) if sodium disappeared from the solution bathing the epithelial side of the skin. When Na^{22} was used, a positive deviation from the flux ratio predicted by Ussing's ('49b) diffusion equation was accepted as evidence that sodium transport was occurring.

The experimental solution used, unless otherwise noted, contained 50 mM. NaCl and 5 mM. KCl, titrated to a pH near 7. The NaCl used was Merk and Co., low K^+ sodium chloride, lot No. J4106. This reagent contained not more than 0.005% KCl, and was the only salt used in the K^+ free studies.

The frog used was *Rana pipiens,* obtained from commercial sources.

RESULTS

1. *The development of the hydrogen ion gradient.* The data in figure 2 and table 1 show that a hydrogen ion gradient develops rather rapidly across the isolated skin in unbuffered solution. The inside or corium side becomes basic, the outside or epidermal side acid. This gradient is held for many hours, changing usually only slightly after the first 30 or 40 minutes.

2. *Effect of phosphate buffers on the potential.* An examination of figure 2 reveals that while the pH of the solution bathing the corium remained fairly constant, the potential did not. It seemed worthwhile, therefore, to repeat the experiments of Meyer and Bernfeld ('46), using their buffer solution inside and tap water outside. The results of such an experiment are shown in figure 3, and further data are given in table 2. As shown in figure 3, only rarely did the potential change in the manner predicted by the H^+ electrode equation.

3. *Effect of aeration in K^+ free solution.* Although only a small amount of acid was produced during transport (fig. 2), it seemed worthwhile to examine the development of the

pH gradient further, in the hope of determining whether any connection might exist between the gradient and the transport process. To carry out such studies, it was decided to work first of all with NaCl solutions, in order to learn whether the gradient depended upon sodium ion or one of the other ions commonly used in Ringer's solution. The results of such an experiment are shown in figure 4.

As shown in figure 4, no transport of Na^+ occurred, although Na^+ did leak from the skin into the solution bathing the corium. It may be further noticed that the solution bathing

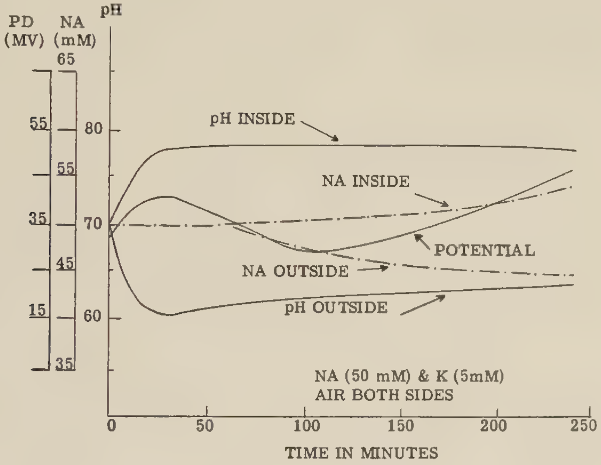

Fig. 2 Changes in sodium ion concentration, hydrogen ion concentration, and potential across the skin as a function of time.

TABLE 1

Rate of development of the sodium and hydrogen ion gradients

FROG	pH START		pH 10 MIN.		pH 20 MIN.		pH 30 MIN.		pH 60 MIN.		Na^+ mM. START		Na^+ mM. 60 MIN.	
	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.
1	7.0	7.0	7.4	7.0	7.5	6.95	7.6	6.9	7.7	6.85	50	50	50	50
2	6.9	6.9	7.5	6.9	7.6	6.8	7.8	6.8	7.9	6.6	50	50	50	49
3	7.0	7.0	7.4	6.95	7.55	6.95	7.7	6.9	7.85	6.85	50	50	50	48
4	7.1	7.1	7.55	6.15	7.6	6.0	7.7	6.1	7.8	6.4	50	50	50	50
5	6.6	6.6	7.7	7.1	7.9	7.0	7.95	7.0	8.15	6.95	50	50	50	51

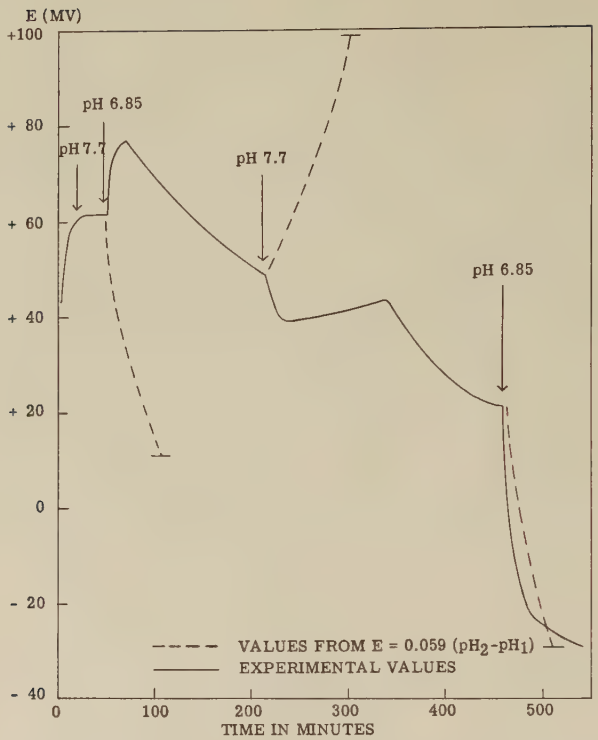

Fig. 3 Changes of potential with changes of the pH of the corium. Tap water outside, phosphate buffer according to Meyer and Bernfeld ('46) inside. The sign of the potential refers to the corium.

TABLE 2

Data showing the changes of potential with changes of the pH of the corium. Calculated values are from the equation, $E = 0.059\ (pH_2 - pH_1)$

FROG NO. 1		FROG NO. 2		FROG NO. 3		FROG NO. 4	
Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
— 50	+ 3	— 50	+ 15	— 50	— 21	— 50	+ 3
+ 50	+ 17	+ 50	— 8	+ 50	+ 3	+ 50	+ 2
— 50	— 21	— 50	— 50	— 50	— 45	— 50	0
+ 50	— 8	+ 50	— 6	+ 50	— 7	+ 50	— 9

the epidermis becomes, at the best, only slightly acid, and is usually slightly basic (table 3).

The finding that the skin could produce base without also producing acid led to the speculation that the skin must carry out different kinds of processes on its two sides, and that these processes might be affected by the oxygen supply. In order to test this idea, experiments of the same kind described in figure 4 were carried out, but only the corium side of the skin was aerated. Figure 5 shows the results of such an experiment.

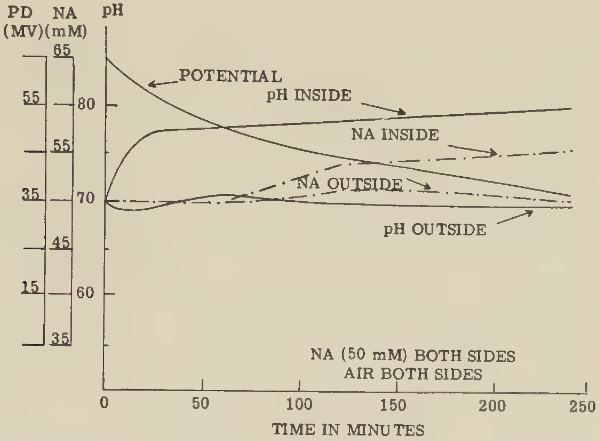

Fig. 4 Development of sodium ion and hydrogen ion gradients in potassium free solutions. Air both sides.

TABLE 3

Data from experiments of the type described in figure 4

FROG	pH START		Na+ START		pH 60 MIN.		Na+ 60 MIN.		pH 180 MIN.		Na+ 180 MIN.	
	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.
1	6.9	6.9	50	50	7.9	7.1	50	50	8.0	7.0	53	51
2	7.0	7.0	50	50	7.8	7.3	50	52	7.7	7.3	54	51
3	7.1	7.1	50	50	7.9	7.2	50	50	8.1	7.4	55	49
4	7.0	7.0	50	50	7.8	7.0	50	50	7.7	6.9	51	48
5	6.4	6.4	50	50	7.6	7.0	53	50	7.7	7.3	54	53

As shown in figure 5, Na^+ does move from the epidermal side to the solution bathing the corium, but only slowly, and there is a slight production of acid on the epidermal side. Further data are given in table 4.

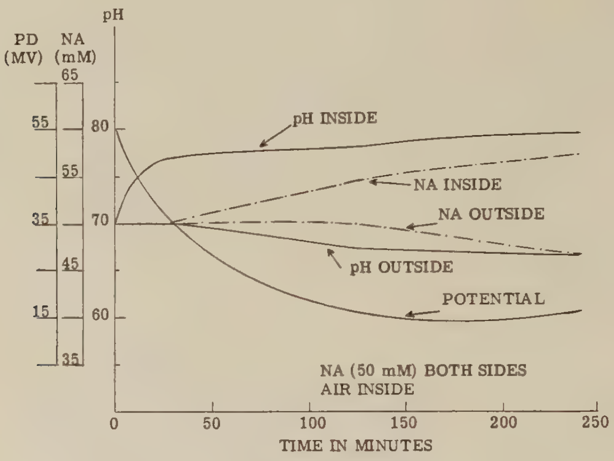

Fig. 5 Development of sodium ion and hydrogen ion gradients in potassium free solutions. Air inside only.

TABLE 4

Data from experiments of the type described in figure 5

FROG	pH START		Na^+ START		pH 60 MIN.		Na^+ 60 MIN.		pH 180 MIN.		Na^+ 180 MIN.	
	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.
1	6.9	6.9	50	50	7.8	6.8	50	48	7.95	6.85	53	47
2	7.0	7.0	50	50	7.75	6.9	53	51	7.85	6.55	55	48
3	7.1	7.1	50	50	7.8	7.1	50	50	7.7	7.05	53	48
4	6.8	6.8	50	50	7.8	6.9	53	48	7.9	6.6	56	45
5	7.0	7.0	50	50	7.95	7.1	51	49	8.2	6.8	53	46

If only the epidermal side was aerated, the production of base was reduced, and no acid appeared in the solution bathing the epidermis. Two skins took up Na^+ from the outside solution, but it did not appear in the solution bathing the corium. Other skins leaked Na^+ from the skin into both solutions. Data are given in figure 6 and table 5.

The results given above would seem to indicate that the production of acid is in some way connected with the transport process. This idea gained support from the results obtained by treating the skin with 0.001 M dinitrophenol. According to Fuhrman ('52) and Levinsky and Sawyer ('52), DNP in this concentration completely blocks transport. As seen in figure 7, both sides of the skin became basic, and no transport of sodium occurred.

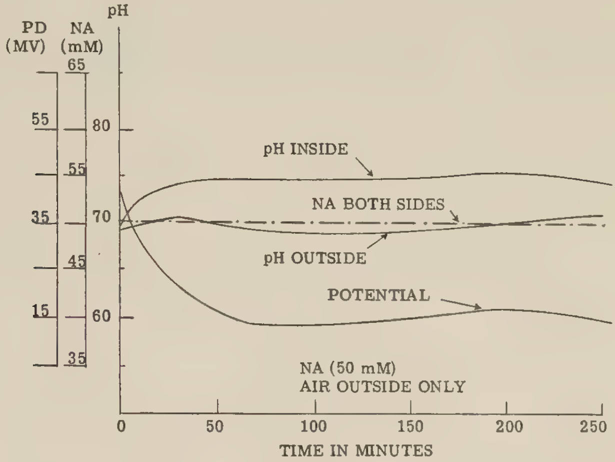

Fig. 6 Development of sodium ion and hydrogen ion gradients in potassium free solution. Air inside only.

TABLE 5

Data from experiments of the type described in figure 6

FROG	pH START		Na+ START		pH 60 MIN.		Na+ 60 MIN.		pH 180 MIN.		Na+ 180 MIN.	
	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.	Ins.	Outs.
1	6.9	6.9	50	50	7.5	7.0	50	49	7.55	7.1	50	46
2	6.6	6.6	50	50	7.4	6.9	51	50	7.4	7.0	53	52
3	7.0	7.0	50	50	7.6	7.1	50	48	7.5	7.0	51	44
4	7.0	7.0	50	50	7.5	6.9	50	51	7.4	7.0	50	51
5	6.8	6.8	50	50	7.6	6.8	50	49	7.6	6.9	53	50

It is possible, however, to demonstrate the appearance of acid in the outside solution without transport taking place.

This may be done by simply cutting down the oxygen supply of the skin by not aerating, so that the only oxygen supply is that originally present in the solution or that which might diffuse in from the atmosphere. The results of such an experiment are given in figure 8.

If one adds K^+, the results are quite different. It apparently makes no difference where the skin is aerated as long as

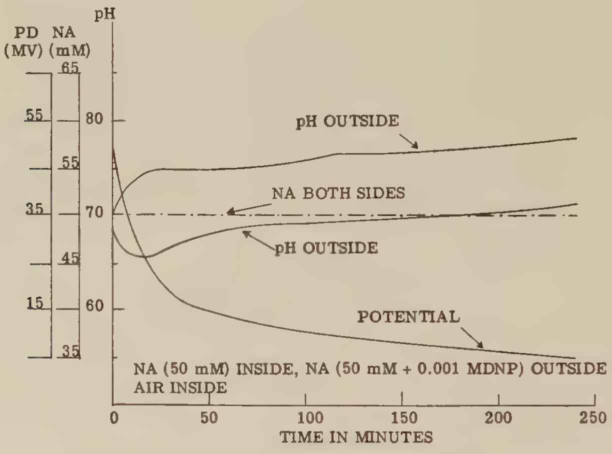

Fig. 7 Effect of DNP on the development of the sodium ion and hydrogen ion gradients.

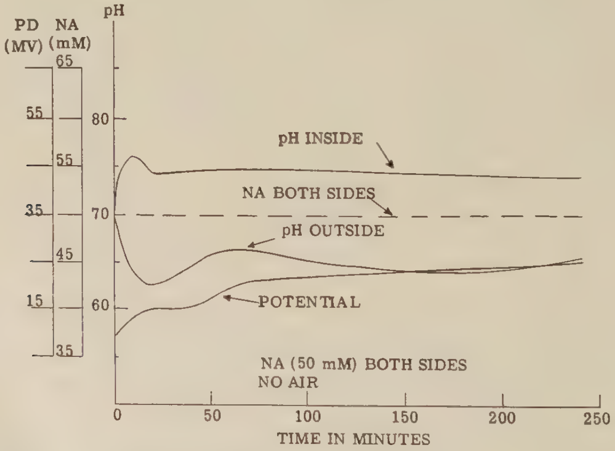

Fig. 8 Effect of oxygen starvation on the development of the sodium ion and hydrogen ion gradients. Potassium free solution.

oxygen is supplied. A good supply of oxygen is necessary, however, for as figure 9 shows, transport does not occur if oxygen is not supplied. Figures 8 and 9 show that the production of base is lower under such conditions.

While the flame photometer failed to demonstrate that Na^+ transport was taking place under some conditions, the possibility remained that such small amounts of Na^+ were moved that it was not detected during the time of the experiment.

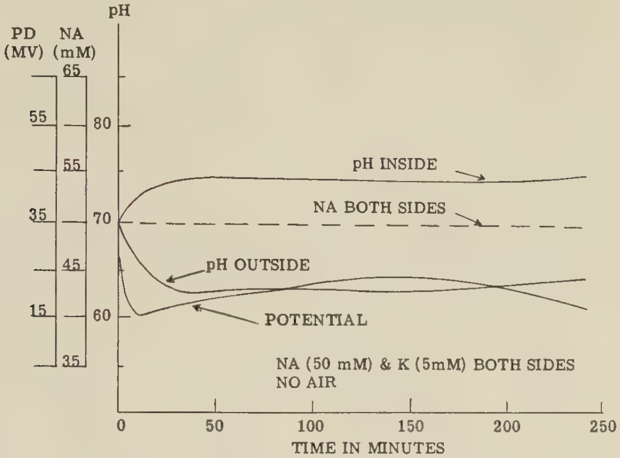

Fig. 9 Effect of oxygen starvation on the development of the sodium ion and hydrogen ion gradients.

Ideally, one should use the double labelling technique introduced by Ussing ('52), but the short half life of Na^{24}, coupled with transportation difficulties, made such a course impractical. It was decided, therefore, to use two adjacent pieces of skin and measure the influx of Na^{22} on one, and the outflux on the other. Huf et al. ('51), using bags formed from the hind legs, found that two bags from the same frog under the same experimental conditions differed in activity by less than 5%, an observation confirmed by Levinsky and Sawyer ('52). By using large Jumbo frogs (60–80 gms) it was possible to obtain enough abdominal skin to supply two matched cells. Usually, the potentials would match within 5% after

TABLE 6

Comparison of the potentials and Na^{22} influxes of two adjacent pieces of abdominal skin. The potentials are in millivolts, the influxes in μeq $Na^{+}/cm^{2}/hr$. The numbers at the top refer to successive hourly flux periods. The bathing solution contained 50 mM NaCl + 5 mM KCl. Air both sides.

FROG	1		2		3		4	
	P.D.	Influx	P.D.	Influx	P.D.	Influx	P.D.	Influx
1a	40	0.44	42	0.47	38	0.52	38	0.56
1b	39	0.45	40.5	0.44	38	0.46	38	0.49
2a	48	1.15	30.5	1.06	33	1.05	33	1.11
2b	48	1.10	29.5	0.99	31	0.95	32	0.93
3a	26	0.89	27	0.96	25	0.91	25	0.97
3b	29	0.87	29	0.91	28	0.85	23	0.86
4a	19	1.35	18	1.42	22	1.21	23	1.05
4b	18	1.31	18	1.33	19	1.29	21	1.19

TABLE 7

Influx and outflux of Na^{22} in K^{+} free solution, air inside only. The experimental ratio exceeded the calculated in frog no. 1, and the last three flux periods of frog no. 4. Sodium values are in μeq/cm^{2}/hr. The p.d.'s in millivolts, and is the average of the two pieces of skin. The top figure refers to successive flux periods.

		1	2	3	4
1	Influx	0.60	0.60	0.53	0.52
	Outflux	0.10	0.10	0.03	0.05
	P.D.	33	33	30	30
	M_1/M_2 calc.	3.7/1	3.7/1	3.3/1	3.3/1
	M_1/M_2 exp.	6/1	6/1	17.5/1	10.4/1
2	Influx	0.45	0.44	0.36	0.29
	Outflux	0.15	0.13	0.16	0.18
	P.D.	30	29	26	22
	M_1/M_2 calc.	3.3/1	3.3/1	2.8/1	2.4/1
	M_1/M_2 exp.	3/1	3.4/1	2.3/1	1.6/1
3	Influx	0.31	0.37	0.33	0.29
	Outflux	0.15	0.12	0.11	0.10
	P.D.	33	31	29	24
	M_1/M_2 calc.	3.7/1	3.4/1	3.2/1	2.6/1
	M_1/M_2 exp.	2/1	3.1/1	3/1	2.9/1
4	Influx	0.32	0.43	0.52	1.08
	Outflux	0.13	0.05	0.10	0.17
	P.D.	32	37	38	38
	M_1/M_2 calc.	3.6/1	4.3/1	4.5/1	4.5/1
	M_1/M_2 exp.	2.5/1	9/1	5.2/1	6.4/1

being in the cells for 30 minutes. A few skins showed widely different potentials and were rejected. It was found, however, that Na^{22} influx usually varied by close to 10% after 4 hours, although the potentials might match closely. For that reason no experiments were continued for more than 4 hours. Data from control experiments are shown in table 6.

Of the first 14 frogs tested in K^+ free solution and with aeration on the corium side only, only 5 transported sodium ion, a different result from that obtained in the summer experiments. Data are given in table 7.

When only the outside of the skin was aerated, 7 of 12 skins transported, a result which had never been obtained previously. Data are given in table 8.

TABLE 8

Influx and outflux of Na^{22} *in* K^+ *free solution. Air outside only. The experimental ratios exceed the calculated in the first three frogs, and in the final flux period of frog no. 4. Flux values in* $\mu eq\ Na^+/cm^2/hr$. *The averaged potential is in millivolts.*

		1	2	3	4
1	Influx	0.46	0.77	0.73	0.74
	Outflux	0.04	0.08	0.16	0.15
	P.D.	22	23	24	24
	M_1/M_2 calc.	2.4/1	2.5/1	2.6/1	2.6/1
	M_1/M_2 exp.	11/1	9.6/1	4.6/1	5/1
2	Influx	0.46	0.42	0.72	0.76
	Outflux	0.01	0.11	0.11	0.05
	P.D.	21	18	18	16
	M_1/M_2 calc.	2.3/1	2/1	2/1	1.9/1
	M_1/M_2 exp.	46/1	4/1	6.5/1	15/1
3	Influx	0.62	0.71	0.77	0.71
	Outflux	0.11	0.08	0.11	0.07
	P.D.	53	49	42	41
	M_1/M_2 calc.	8/1	7/1	5.4/1	5.1/1
	M_1/M_2 exp.	6/1	9/1	7/1	10/1
4	Influx	0.34	0.31	0.36	0.61
	Outflux	0.14	0.05	0.12	0.13
	P.D.	57	57	48	37
	M_1/M_2 calc.	10/1	10/1	6.8/1	4.4/1
	M_1/M_2 exp.	2.4/1	6/1	3/1	4.7/1

In no case, however, did transport occur after the first hour when the skins were aerated on both sides. Data are given in table 9.

It was also found that sodium transport could be blocked, or initiated, by aerating on one side of the skin and then changing the air supply to the other side. Data are given in table 10.

TABLE 9

Influx and outflux of Na^{22} in K^+ free solution. Air both sides. Na^{22} values in $\mu eq/cm^2/hr$. Potential in millivolts

		1	2	3	4
1	Influx	0.16	0.18	0.34	0.39
	Outflux	0.05	0.18	0.16	0.15
	P.D.	17	25	25	25
	M_1/M_2 calc.	2/1	2.7/1	2.7/1	2.7/1
	M_1/M_2 exp.	3/1	1/1	2.1/1	2.6/1
2	Influx	0.22	0.26	0.37	0.33
	Outflux	0.07	0.09	0.15	0.16
	P.D.	29	26	25	19
	M_1/M_2 calc.	3.3/1	2.8/1	2.7/1	2.1/1
	M_1/M_2 exp.	3.1/1	2.9/1	2.5/1	2/1
3	Influx	0.08	0.29	0.42	0.59
	Outflux	0.03	0.09	0.07	0.11
	P.D.	52	52	47	44
	M_1/M_2 calc.	7.9/1	7.9/1	6.5/1	5.7/1
	M_1/M_2 exp.	2.6/1	3.2/1	6/1	5.4/1

The "matched skin" technique would seem to offer an opportunity to study the role of K^+ in maintaining the transport process, i.e. by following the influx and outflux of Na^{22} in the presence and absence of K^+. This proved to be difficult, because while the skins might match very well when mounted, the potentials soon separated, with the skin in K^+ free solution having the higher potential. Also, it was not possible to know which side of the skin in K^+ free solution should be aerated. Data are given, therefore, only on skins in which the influx was fairly high in K^+ free solution. As shown in

TABLE 10

Na^{22} influx and outflux in K^+ free solution. In all cases, one side of the skins was aerated on one side for the first two flux periods, then the air was switched to the other side. Frogs nos. 1 and 2 were aerated first on the corium side and the flux ratio increased after the air was switched. Frogs nos. 3 and 4 were first aerated on the epidermal side, and switching of the air reduced the flux ratio. In frog no. 5, the experimental flux ratio always exceeded the calculated, and in frog no. 6, the experimental flux ratio was always less than the calculated. Frogs nos. 5 and 6 were first aerated on the epidermal side.

		1	2	3	4
1	Influx	0.11	0.16	0.36	0.36
	Outflux	0.11	0.15	0.07	0.02
	P.D.	16	20	26	36
	M_1/M_2 calc.	1.9/1	2.2/1	2.8/1	4.2/1
	M_1/M_2 exp.	1/1	1.1/1	5/1	18/1
2	Influx	0.32	0.36	0.52	0.43
	Outflux	0.13	0.09	0.10	0.05
	P.D.	32	37	38	37
	M_1/M_2 calc.	3.6/1	4.3/1	4.5/1	4.3/1
	M_1/M_2 exp.	2.5/1	4/1	5.2/1	8.6/1
3	Influx	0.34	0.31	0.36	0.61
	Outflux	0.14	0.05	0.12	0.10
	P.D.	57	57	48	37
	M_1/M_2 calc.	10/1	10/1	6.8/1	4.4/1
	M_1/M_2 exp.	2.4/1	6/1	3/1	6/1
4	Influx	0.89	0.82	0.89	1.07
	Outflux	0.12	0.11	0.09	0.13
	P.D.	56	55	48	41
	M_1/M_2 calc.	9.2/1	9/1	6.8/1	5.1/1
	M_1/M_2 exp.	7.4/1	7.4/1	9.9/1	8.2/1
5	Influx	0.73	1.40	0.92	0.90
	Outflux	0.06	0.04	0.14	0.19
	P.D.	31	33	33	33
	M_1/M_2 calc.	3.4/1	3.7/1	3.7/1	3.7/1
	M_1/M_2 exp.	12/1	35/1	6.6/1	4.9/1
6	Influx	0.14	0.21	0.29	0.19
	Outflux	0.04	0.11	0.12	0.12
	P.D.	37	38	38	38
	M_1/M_2 calc.	4.5/1	4.5/1	4.5/1	4.5/1
	M_1/M_2 exp.	3.7/1	2/1	2.5/1	1.5/1

TABLE 11

Comparison of potentials and Na^{22} influxes in matched skins. The "a" skins were in K^+ free solution. Na^{22} values in μeq/cm²/hr., potentials in millivolts. All skins were aerated on the epidermal side.

	P.D.	INFLUX	P.D.	INFLUX	P.D.	INFLUX
1a	22	0.78	22	0.61	22	0.29
1b	16	0.56	17.5	0.66	18	0.77
2a	44	0.29	40	0.37	41	0.28
2b	38	0.28	34	0.39	31	0.52
3a	28	0.63	33	0.55	37	0.29
3b	27	0.69	24	0.71	19	0.93

TABLE 12

Outflux of matched skins. Comparison of potentials and Na^{22} outflux. The "a" skins were in K+ free solution. Na^{22} values in μeq/cm²/hr., potentials in millivolts. Skins 1 and 2 were aerated outside only, skin 3 inside only.

	P.D.	OUTFLUX	P.D.	OUTFLUX	P.D.	OUTFLUX
1a	35	0.21	33	0.31	32	0.19
1b	38	0.11	31	0.05	29	0.07
2a	32	0.08	34	0.17	33	0.18
2b	32	0.09	28	0.08	24	0.12
3a	43	0.06	47	0.11	49	0.13
3b	41	0.05	36	0.03	38	0.08

tables 11 and 12, two effects of K^+ free solutions are apparent — a steadily decreasing rate of influx (table 11) and a high rate of outflux (table 12), as compared with the controls.

DISCUSSION

From the data presented in figure 2 and table 1, it seems clear that, in unbuffered solution at least, there is no appreciable exchange of sodium ion for hydrogen ion. Inasmuch as the hydrogen ion gradient is usually fully developed before any detectable amount of sodium has moved, and as the gradient remains fairly constant for hours, it seems fair to conclude that sodium transport does not involve an exchange for hydrogen ion (Ussing, '48, '49a; Huff and Wills, '53). Indeed, it appears that hydrogen ion is ejected by the skin only until sodium transport starts, very little hydrogen ion appearing after the first 30 minutes. It is of interest to note that while sodium transport has not been observed without the previous appearance of hydrogen ion (figs. 2 and 5), it is possible to demonstrate the appearance of hydrogen ion without sodium transport taking place (figs. 8 and 9). Further, while the amount of base appearing depends only on the supply of air to the corium (figs. 2, 4, 6, 8 and 9), the appearance of hydrogen ion depends upon the site of aeration in K^+ free solution, and seems to be enhanced if air is not supplied at all (figs. 4, 5, 6 and 8). It also seems clear that the hydrogen ion gradient is the first gradient to appear across the skin (except the electrical gradient), and, in terms of concentration ratios, is much greater than the sodium gradient. At the present time it is not possible to evaluate the importance of the gradient in the overall transport process, other than to state that the hydrogen ion gradient always precedes sodium transport, and that sodium transport has never been observed in the absence of the gradient.

Contrary to the findings of Meyer and Bernfeld ('46), it has not been possible to demonstrate any relationship between the pH of the corium and the potential difference across the skin (fig. 3, table 2). Meyer and Bernfeld ('46) report that thick-skinned winter frogs responded slowly to pH changes, but as these experiments were carried out in the spring and summer months, the failure to confirm their results cannot be explained on the basis of a seasonal difference. The reason it has not been possible to repeat their experiments is not known.

While it is known that low potassium solutions inhibit or greatly decrease sodium transport (Huf and Wills, '51; Ussing, '54; Huf, Wills and Arrighi, '55), the mechanism by which potasium maintains transport is not known. Huf and Wills ('51) suggested that potassium might be an important element in the mosaic of cellular structure and thereby regulate permeability, or that it might act as an activator for the enzymes which carry out the transport process. In a later paper (Huf and Wills, '53), it was suggested that potassium ion might regulate chloride permeability. Recently Huf has shown that in potassium free solutions intracellular potassium is exchanged for extracellular sodium (Huf et al., '55), and stressed the possible role of the mitochondria in potassium adsorption and sodium transport.

The data presented here offer some support for the earlier suggestions of Huf and Wills ('51). As shown in table 12, the outflux of Na^{22} was consistently higher in the skins immersed in potassium free solution. The same phenomenon is seen in the data of Ussing ('54). Potassium ion also prevents the leakage of sodium from the skin into the bathing solution (figs. 2, 4 and 5). Surprisingly, the potentials were somewhat higher in the potassium free saline than in the controls (tables 11 and 12), although this situation might well have been reversed had the experiments been extended for a few more hours. The recent paper of Huf et al. ('55) also shows the potentials of the skins kept in potassium free dilute saline to be higher than skins bathed in dilute saline containing potassium.

The data in table 11 show that Na^{22} influx decreased with time in the K^+ free solutions, probably due to an exchange of intracellular potassium for extracellular sodium (Huf et al., '55). Such a change of the K^+/Na^+ ratio might well have profound effects on the metabolism of the tissues involved. In this connection, it is of interest to recall the work of Utter ('50) with rat brain extracts. It was found that sodium inhibited, and potassium (and ammonium ion) stimulated glycolytic activity when glucose was the initial substrate. According to Utter ('50), sodium had at least three effects: (1) the stimulation of the conversion of A.T.P. to A.M.P. by apyrase; (2) the inhibition of phosphorylation of A.M.P.: and (3) a decrease rate of removal of A.M.P. by phosphoenolpyruvate kinase which is also inhibited by sodium. Hers ('51) has shown that fructokinase requires potassium and is inhibited by sodium. Hastings et al. ('55) reported that sodium replacement of potassium in rat liver slices produced very significant changes in their carbohydrate metabolism. Of especial interest is a report of Boyer et al. ('43). Using rat brain extract, it was found that while the rate of oxidation was not affected by the absence of potassium, the ion was necessary if phosphorylation was to accompany oxidation. Both inorganic phosphate and sodium ion inhibited the transfer of phosphate from 3-phosphoglycerate to creatine. Here then, is one place where potassium loss would interefere with oxidative phosphorylation. It must be pointed out, however, that there is no evidence that this particular metabolic step is involved in the actual transport of sodium. It does seem reasonable to suppose, however, that at least part of the effects observed in potassium free solutions are due to an altered metabolism in the skin.

The finding that the transport of sodium in potassium free solution is dependent upon the site of aeration is surprising. In the earlier experiments, carried out in the spring and summer months, aeration of the epidermal side invariably blocked transport (figs. 4 and 6). In the Na^{22} experiments, which were carried out in the winter months, the situation was somewhat different, i.e. in some cases aeration of the corium side blocked transport (tables 7 and 10). The earlier experiments were carried out with the flame photometer, and it is possible that transport was occurring in small amounts without being detected. As Ussing ('49b) has pointed out, it is necessary to know the ratio of the ion fluxes before one can be sure that active transport is taking place.

It may also be that the reversal noted is peculiar to the large females used, or that it is a seasonal difference. Such differences have been reported by some workers but have not been seen by others. Bayliss and Bradford ('86) reported that the direction of the change of the resting potential obtained by stimulation of the skin nerves depended upon the time of year that the experiments were carried out. According to them, a negative "varslag" was obtained during the breeding season; otherwise the potential increased upon stimulation. Seasonal differences have also been reported by Williams and Sheard ('32) and Francis and Pumphrey ('33) among others. However, Boell and Taylor ('33), Gatty ('41), and Huf et al. ('55), among others, have not observed seasonal differences. When one considers the variation among different shipments of frogs, it is difficult to say whether seasonal variations occur or not. As a general impression, it would seem that frogs received in the late fall and winter months are not as healthy as the spring and summer frogs. It has been noted, however, that the outfluxes in potassium solutions of the frogs run in December and January are two to 5 times greater than frogs run under the same conditions in September and October (unpublished experiments). Further experiments will be necessary before it can be decided whether this is a seasonal or sexual difference, or perhaps both.

At least a partial explanation of the aeration effect may be found in observations made by Hoagland and Rubin ('36) and Rubin and Syrocki ('36). According to the latter authors, potassium ion is concentrated primarily in the epidermal layers of the skin, only small amounts occurring in the corium. Hoagland and Rubin ('36) reported that potassium was released from the skin by mechanical stimulation from an air jet. While the air bubbles do not actually touch the skin in the chamber used, they do pass by rather closely and set up a strong circulation of water, and, conceivably could accelerate the loss of potassium from the skin. If this is correct, then it would be necessary to postulate that, in some frogs at least, there is a shift of potassium from the epidermal to the corium layer, and, at the moment, no evidence for such a shift exists. Neither would such an explanation account for the data in table 10, where it is seen that transport could be either stopped or started by switching the site of aeration. Thus, along with the loss of potassium which undoubtedly occurs, it would seem that a high oxygen tension in the proper location inhibits transport in the absence of external potassium, perhaps by a reverse Pasteur effect. While it would not seem likely that oxygen would have any difficulty passing through the skin, the low production of base shown in figure 6 indicates that this may be the case.

The possibility also remains that potasium ion may be involved in the production or consumption of a redox couple which is involved in the pump mechanism. However, no clear evidence exists that a redox system is involved, although the presence of the hydrogen ion gradient is suggestive. The evidence recently reviewed by Conway ('55) does not seem to be decisive. It is hoped that experiments now under way, using reduced oxygen pressures, will shed further light on this problem.

Finally, comment is required on some of the data presented in tables 7, 8, and 9. It is seen that in some cases the ratio of fluxes of sodium ion equals, or is less than, the ratio of fluxes expected from an innocuous ion moving under the influence of an electrical gradient (Ussing, '49b), i.e., that the movement of sodium could be explained on the basis that it is being dragged through anion movement. Whenever the ratio of fluxes did not exceed the calculated ratio, it was not possible to decide whether or not transport was taking place, and therefore the statement is made that transport did not occur. It is recognized that such a procedure is open to criticism, and may well not be correct. Rosenberg ('54) has pointed out that active transport should not be defined in terms of deviations from diffusion equations, but should be defined as the movement of a substance from a lower to a higher electrochemical potential. Such a definition, while no doubt formally correct in this case, is difficult to use in practice. It is not intended, at this time, to discuss the relationship between ion transport and the presence of the potential; however, it is curious that under some conditions the sodium flux follows the ratios calculated from Ussing's ('49b) diffusion equation.

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SUMMARY

1. Using unbuffered solutions, it has been found that the solution bathing the corium side of the isolated frog skin becomes basic, the epidermal side acid. The amount of acid produced is too small to account for any appreciable movement of sodium ion by an hydrogen exchange mechanism.

2. The hydrogen ion gradient is the first gradient developed across the skin, and in terms of concentration ratios is much larger than the sodium gradient. After 30–40 minutes the hydrogen ion gradient remains fairly constant. Sodium transport was not observed in the absence of the hydrogen ion gradient.

3. In potassium free solutions, the epidermal side of the skin did not become acid if the skin was aerated on that side. The production of base was found to be independent of potassium ion, and depended only on an adequate air supply to the corium side of the skin.

4. When air was not supplied, the production of base was reduced, the epidermal side became acid, and sodium transport did not occur.

5. When the influx and outflux of two adjacent pieces of skin were compared, one being in potassium free solution, it was found that the absence of potassium led to higher potentials, a decreasing rate of sodium influx, and an increasing rate of outflux. The flux ratios of skins in potassium free solution could be altered by changing the site of aeration.

6. In summer frogs, it was found that sodium transport occurred in potassium free solution only if aerated on the corium side. In winter frogs, aeration on the corium side often inhibited transport.

7. It is suggested that a shift of extracellular sodium for intracellular potassium might alter the permeability properties of the skin and skin metabolism.

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STUDIES ON REGRESSION AND REGENERATION IN THE URODELE FORELIMB AFTER LOCALIZED ULTRAVIOLET RADIATION[1]

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FIVE FIGURES

Regeneration of parts after amputation, and the development of supernumerary structures after various experimental treatments, are characteristic of the larvae of urodele amphibia. In the case of the limb, both phenomena are ordinarily preceded by some regression of the tissues involved. An earlier paper (Butler and Blum, '55) described regeneration of supernumerary appendages of the elbow region of the forelimb of *Amblystoma,* after exposure of that area to ultraviolet radiation. In the present paper we are concerned with quantitative studies of regression of the forearm, caused by localized irradiation with ultraviolet light, and with regeneration, after amputation in the irradiated area. Photorecovery, brought about by illumination with "visible" light immediately after the ultraviolet radiation has been included in our study.

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MATERIAL AND METHODS

We have used the larvae of two species, *Amblystoma opacum* and *A. punctatum,* finding no differences in their response with regard to ultraviolet radiation and subsequent photorecovery, other than possible minor quantitative ones. These animals were obtained from either North Carolina or the New Jersey area; at the time of exposure they were from 18 to 27 mm, over-all length, but in a given experiment the range was usually considerably less than this. The irradiation of adequate numbers of animals may require several weeks during which they are actively growing, and in order to obtain uniformity at the time of irradiation it is necessary to select animals of approximately the same size from among a larger group. Selection was usually made on the basis of uniformity in the dimensions of the forearm, since this is important as regards uniformity of penetration of the radiation into the limb, rather than on total body length. Measurements were made under anaesthesia, using a 1:10,000 solution of MS 222; measurements of limb length were made to the nearest 0.1 mm using a low power microscope with calibrated ocular micrometer; total length was measured to the nearest 0.5 mm by comparison with a millimeter scale.

The larvae were exposed to doses of ultraviolet radiation, measured as described below, with the animal lying on its back on a cushion of black nylon fibers in an aluminum dish 5.5 cm in diameter and 1 cm deep, blackened inside. The areas not to be exposed were covered with aluminum foil blackened on the under surface. Generally 4 animals were placed in the same dish, oriented so that the parts to be irradiated were contained within a circle about 3 cm in diameter. After placing and shielding the 4 animals the dish was covered with a shield blackened on the underside, having an opening just large enough to admit radiation to the area desired. This method of shielding reduces scattered radiation to a minimum; our experimental results indicate that the amount of ultraviolet reaching the shielded parts was negligible. This orientation of the animals insures that all parts exposed receive very nearly the same dose of incident radiation.

In our earlier experiments no attempt was made to control the temperature during exposure, since it could be assumed that the basic photochemical reactions are essentially independent of temperature. It was found, however, that on warm days the temperature sometimes rose to a point where injurious effects might be anticipated. To avoid these, the metal mounting on which the dish containing the animals rested during the exposure, was equipped with a copper coil through which water circulated from a refrigerated bath. By this device it was possible to keep the temperature in the dish between 18 and 21°C.

The method of dosage with ultraviolet light was essentially the same as that used by Blum, Kirby-Smith and Grady ('41) (see also Rieck, '54) in the study of carcinogenesis in mice. It employs the radiation from an intermediate pressure (atmospheric pressure) mercury arc (Hanovia "Analytical" type), monitored by a photocell with titanium element (Westinghouse Electric: WL-767) by means of an integrating counter. These photocells are sensitive only to wavelengths shorter than about 0.32 μ, and hence are not influenced by ordinary room illumination. The monitoring is made feasible by the stability of this type of source as regards the relative intensities of the mercury lines, which do not alter greatly even when the total intensity undergoes considerable variation with voltage and temperature. In practice we operate on the local line voltage, which fluctuates within a certain range, but since dosage is controlled in terms of the integrated response of the photocell, our values do not reflect this fluctuation; this practice also eliminates errors due to the aging of the lamp unless allowed to go too far.

The monitoring photocell is calibrated in terms of a similar one, placed at the plane of the animals, which has been calibrated against a low pressure mercury arc (85% of total radiant flux assumed to be in the 0.2547 μ line), which in turn is compared with a standard carbon filament lamp calibrated by the National Bureau of Standards. The dosage delivered at the plane of the animals is given in this paper in terms of total energy of wavelengths 0.3130 μ and shorter, estimated from the spectral characteristics of the photocell, and of this type of mercury arc. Both spectral measurements were made some years ago at the National Cancer Institute (see Blum, Kirby-Smith and Grady, '41), the relative values obtained being in good agreement with those now supplied by the manufacturers.

It is obvious that the absolute values given may be considerably in error, easily as much as ± 30%, since each of the calibration steps involves errors of measurement and certain assumptions have to be made. But the relative values expressed in the reproducibility of results are subject to much less error. Thus within a given experiment, based on a given lamp and a given calibration, it is doubtful if the individual measurements are in error by as much as ± 5%, which should be less than that involved in the placing and shielding of the animals. Although it is probable that the reproduction of conditions has varied considerably from experiment to experiment over the years we have been using this method, in the present studies it is doubtful that the total relative error in reproduction of dose from one experiment to the next has been greater than ± 15%; general reproducibility of the biological responses obtained indicate that the error is no greater than this.

As stated above, the intensity of the radiation has not been maintained constant. It has varied considerably ranging at the plane of the animals around 5×10^4 ergs cm^{-2} sec.^{-1} of radiant energy of wavelengths 0.313 μ and shorter. At this intensity it takes about one hour to deliver the highest dose 1.85×10^8 ergs cm^{-2} used in any of these experiments.

After irradiation the animals were removed immediately to thermostated cabinets where the temperature was maintained at 20 ± .5°C. In order to study photorecovery half the animals (two from each group of 4 irradiated) were placed in an illuminated cabinet, the other half in a dark cabinet. After two days the animals in the illuminated cabinet were removed to the dark cabinet, since photorecovery is virtually complete within 48 hours (Blum and Mathews, '52). The illuminated cabinet has a capacity of 0.15 m^3 and is white walled. Two 15-watt "daylight" type fluorescent lamps are placed 12 cm above each of 4 shelves on which rest the glass dishes containing the animals. The intensity of the light in the cabinet has not been measured; but that it is reasonably uniform is indicated by the uniformity of photorecovery among the animals of a given experimental group.

For convenience in the following discussion, the term *irradiation* will refer to the exposure to ultraviolet light, the term *illumination* to subsequent exposure to "visible" light for two days. Since the animals receive during irradiation a certain amount of light that can cause photorecovery (mercury lines at 0.365 μ, and in the violet and blue) a distinction between "illuminated" groups of animals, and "dark" groups is in all cases a relative one.

Experiments designed for different purposes will be referred to in the following pages according to the letters (e.g., WA) by which they were designated in our notes.

GROWTH AND REGRESSION OF THE LIMB

Measurements of the length of the forelimb have been made in many of our experiments, to serve as quantitative indices of growth and regression. Our experiment WA on *A. punctatum* is one of the most complete in this respect and illustrates the general course of events. Two measurements of length were made: (1) the upper arm from shoulder to elbow; and (2) the forearm from elbow to base of digits. Initial measurements of total body length from snout to tail were also recorded. Larvae were selected for measurement and irradiation when the limb had reached appropriate dimensions; thus, although the irradiations spread over a period of 20 days, the animals represented a reasonably uniform pool as regards limb dimensions at the beginning of the experimental treatment. The initial measurements for the 212 larvae used were: average over-all length 23.3 mm (S. D. 0.66 mm); average length of right upper arm 1.74 mm (S. D. 0.095 mm); average length of right forearm 1.76 mm (S. D. 0.091 mm).

The growth of normal limbs *not exposed* to ultraviolet radiation is represented in figure 1. The initial values (at zero time in the figure) are the averages given above of measurements on the right limbs of 212 animals. Within 24 hours after these measurements were made, the right forearm of every animal from the elbow to the tips of the digits, was exposed to ultraviolet radiation (for reasons of feasibility about half were measured the day before irradiation, half the same day). Following the initial measurement and irradiation, the animals were divided into 6 groups which were treated according to the plan in table 1. Thus each value in figure 1 after the first one, is the average of measure-

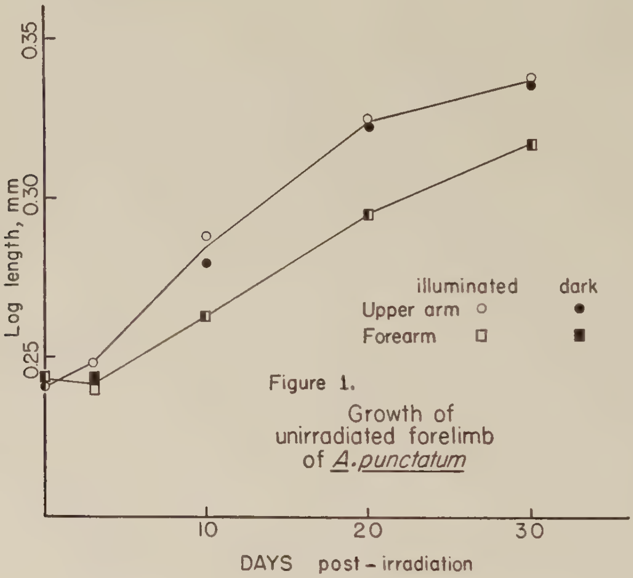


Figure 1

ments on the unirradiated left limbs of the 34 to 36 larvae in one of the groups, as the table indicates; the measurements at three days after irradiation were made on groups 1 and 2; those at 10 days on groups 3 and 4; and those at 20 and 30 days on groups 5 and 6. It is to be pointed out that, although the initial measurement was made on the right arm, the subsequent measurements (for growth of the normal limb) were made solely on the left arm. It is unlikely that this would introduce important error.

TABLE 1

Plan of experiment WA

(A. punctatum)

GROUP	NO. OF ANIMALS		ILLUMINATION	TIME OF MEASUREMENTS	TIME OF AMPUTATION
	Start	End			
				(days after irradiation)	*(days after irradiation)*
1	36	36	+	3	3
2	36	36	—	3	3
3	36	36	+	10	10
4	34	34	—	10	10
5	35	35	+	20 and 30	30
6	35	32	—	20 and 30	30
Totals	212	209			

After the first three days, during which there may be a slight depression of growth, possibly a result of the manipulation attendant upon irradiation, growth appears to be continuous, although the relative rate is not constant nor parallel in the upper and lower arm (fig. 1). The most important thing with regard to the present data is that there is no significant difference in the growth of the unirradiated limbs of animals in the dark as compared to those illuminated. Thus the length of the unexposed arm provides a valid datum for comparison with the length of the arm exposed to ultraviolet radiation, whether the animals were illuminated or kept in the dark after the exposure.

The irradiation of the right forelimb consisted of an exposure to 6.2 × 10^7 ergs per square centimeter of ultraviolet radiation of wavelengths 0.313 μ and shorter, applied to the hand and the forearm up to the elbow, as shown in the diagram in figure 2. Immediately afterward groups 1, 3 and 5 were placed in the illuminated chamber for two days, after which they were placed in the dark chamber for the remainder of the experiment. Groups 2, 4 and 6 were maintained in the dark chamber beginning immediately after the irradiation. Thus, the only difference between the light and dark groups was that the latter received two days illumination immediately following the exposure to ultraviolet radiation. Figure 3 shows the changes in length of the forearm following the irradiation. It is apparent that the irradiated limb is shortened

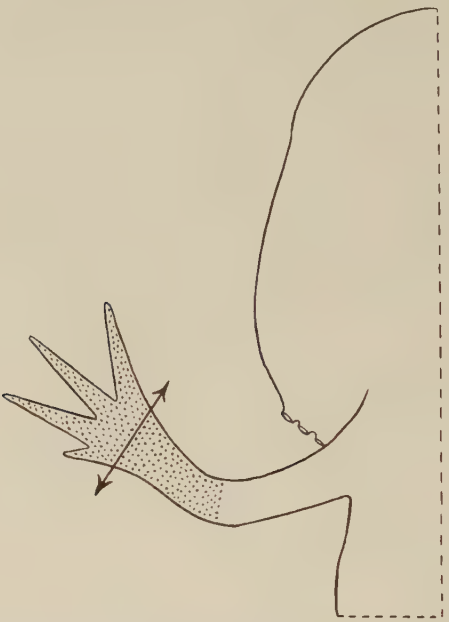
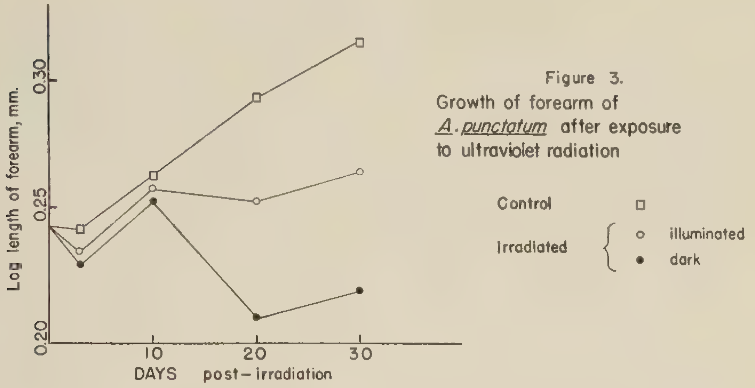
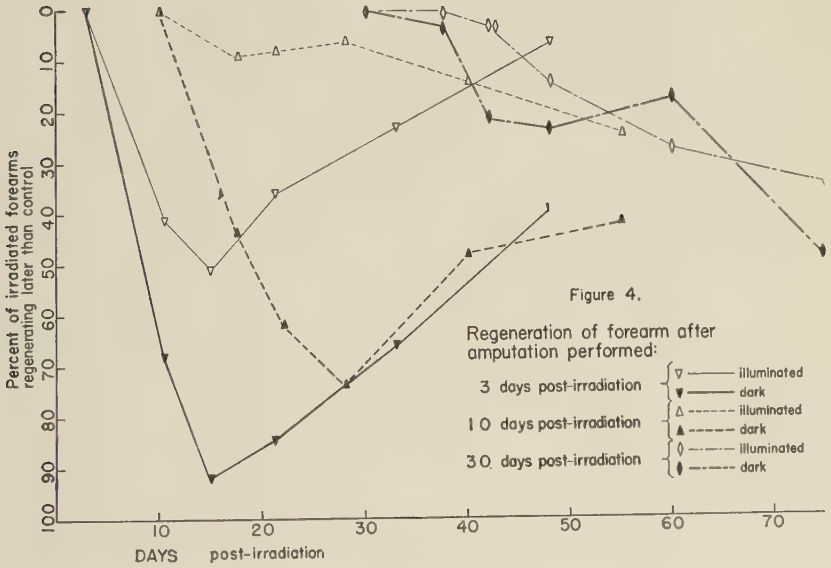


Fig. 2 Irradiation of the right forearm. Animal lies on its back. Stippled area represents region exposed to radiation; remainder of animal was shielded. The double-pointed arrow indicates level of amputation, which was at the distal end of radius and ulna. (See footnote 3.)

relative to the control limb, and that the limbs of the animals maintained in the dark chamber are shortened more than those of the animals placed for two days in the illuminated chamber. Regardless of what interpretation we give these curves, they indicate the importance of making observations at appropriate times; up to 10 days the differences in limb length are slight, but after 10 days they increase rapidly until at 20 days they are very marked.





Figures 3 and 4

As plotted on the logarithmic scale in figure 3, the slopes of the curves indicate the average relative rate of growth in length of the forelimb during the periods between measurements; both positive and negative slopes are seen. Minor differences in length could result from changes, for example, in permeability, which would lead to swelling or shrinking of the limb by exchange of water; from muscular contracture; or possibly from other causes; thus giving positive or negative slope to the curves for short periods of time. But marked, prolonged positive growth rates must indicate accumulation of material, and to a large extent reflect the rate of cell division; whereas negative growth rates of any magnitude must indicate active removal of material, presumably dead cells for the most part. Positive growth, indicating that active cell proliferation is dominant, goes on in the irradiated limbs between three and 10 days, and again between 20 and 30 days; although the ratio of limb length to that of the controls is very different during these two periods. The marked negative growth rate between 10 and 20 days, must, it seems, be dominated by active resorption of dead cells. The apparent small decreases in length during the first three days are more difficult to explain. The differences are not statistically significant, but are in the direction that might be expected, i.e., least in the control limb and greatest in the irradiated limb which was not subsequently illuminated. By analogy to other cells and tissues a transitory decrease in rate of cell division is to be expected, but this would not result in negative growth. On the other hand, while there is probably some sloughing of epidermis during this period (see, Butler and Blum, '55), this could hardly account for much shortening of the limb. The accompanying depression of growth of the control limb suggests some general change stemming from the manipulation during the irradiation. Until further experiment demonstrates the contrary, it seems reasonable to treat these initial changes as trivial, and to disregard them in considering the greater subsequent ones which concern other aspects of these experiments.

Between three and 10 days, the growth curves for the irradiated limbs (both illuminated and not illuminated) have nearly the same slope as that of the control limb; indicating that cell proliferation is taking place at about the same rate in all three (the small differences in length could be persistent from the effect of either actual shortening of the limb or the slowing of cell division during the first three days). Active resorption of any magnitude should result in a decrease of growth rate, and since there is no evidence of this during the 7 to 10 days post-irradiation, it may be assumed that, although many of the cells have been killed or rendered moribund by the ultraviolet radiation, they are not resorbed during this period. Most of the active resorption seems to take place during the period from 10 to 20 days post-irradiation, following which positive growth is resumed by the irradiated limbs at about the same rate as the control limb. The greater negative growth rate and greater shortening of the irradiated limbs of the animals kept continually in the dark, as compared to those illuminated for two days, indicates a greater resorption of tissue in the former. From this we may reason that the illumination for two days results in a smaller number of cells that ultimately die and are resorbed, which agrees with findings for lethal effects of ultraviolet radiation on bacteria and fungi, where the number of survivor cells is greater if the cultures are illuminated after exposure to ultraviolet radiation.

Without going into detail, mention should be made of regressive changes which occur in the distal end of the upper arm after irradiation of the forearm as carried out in Experiment WA. Post-irradiation measurements of the length of the upper arm were made at the same time as those of the forearm and the results plotted. A slight initial negative growth is suggested during the first three days, but after this resorption is apparently never extensive enough to give a negative growth curve, as in the forearm between 10 and 20 days post-irradiation. However, at 10, 20 and 30 days the growth of the upper arm lags behind that of the control; the effect is greater in the dark, less in the illuminated animals. These small changes no doubt result from a small amount of ultraviolet radiation that reaches the distal end of the upper arm, since it is not possible to place the shield so as to expose the whole of the forearm without, in most cases, exposing a small distal portion of the upper arm; and in any case there must be a certain amount of radiation scattered beyond the edge of the shield. Hence, it should not be concluded that changes in the upper arm result from the effects of ultraviolet radiation on the forearm.

TABLE 2

Experiment OH (A. opacum)

Right hand, including wrist, exposed to ultraviolet radiation

NUMBER OF ANIMALS	DOSE ergs cm^{-2}, ($\lambda = .313\ \mu$ and shorter)	RELATIVE GROWTH			
		$\left[\frac{\text{Length 22–23 days after irradiation}}{\text{initial length}}\right]$			
		FOREARM		UPPER ARM	
		Illuminated	Dark	Illuminated	Dark
8	1.67×10^8	1.19	1.05	1.33	1.30
8	0.55×10^8	1.28	1.09	1.32	1.33
7	0.18×10^8	1.32	1.22	1.39	1.37

Dosage and regression. This material is obviously not such that quantitative dosage relationships are likely to reveal essential photochemical mechanisms. In addition to difficulties of experimentation, the amount of radiation required to produce a given amount of regression depends not only upon the area exposed but also on the degree of penetration, which is only roughly known, and must vary with the thickness of the tissue concerned. Absorption is so high that action spectra can have little quantitative meaning. Recognizing the various difficulties, and hence the small amount of pertinent information to be gained, it has not appeared worthwhile to make dosage studies beyond those needed for purposes of orientation. Table 2 will serve as an example; it presents data from experiment (OH) on *A. opacum* in which the right hand as far proximal as the joint between carpals and radius and ulna was exposed to ultraviolet radiation of three dosages. The irradiated limb is compared to the normal control at 22 to 23 days post-irradiation. The forearm, which was exposed at its distal end in the region of the wrist, is increasingly affected as the magnitude of the dose is increased. Photorecovery is indicated by lesser effects on the limbs of the illuminated animals. The upper arm, no part of which was exposed to the radiation, shows no significant variation in length with dose or illumination. The results from other experiments are similar but less complete; in all pertinent cases regression was greater with greater dose, and photorecovery always evident.

REGENERATION AFTER AMPUTATION

Our experiment WA, already discussed with regard to regression, was designed to provide a comparison of the regenerative capacity of the irradiated limb with that of the normal. At different intervals after irradiation, the forearm, of both normal and irradiated limbs, was amputated at the level indicated by the double-pointed arrow in figure 2 (distal ends of radius and ulna).[3] Amputations were performed on different groups of animals, three, 10 and 30 days post-irradiation, according to the schedule given in table 1. Half of the animals were kept in the dark chamber; the other half spent a period of two days in the illuminated chamber immediately after irradiation and were then transferred to the dark.

Comparison of the stage of regeneration of irradiated and normal limbs was made at intervals up to 45 days post-amputation. These comparisons were based on observation as to whether a regeneration blastema had been established, how large it was and whether digits had begun to appear (see, Butler and Blum, '55). Such observations should reflect any differences in the rate of regeneration in the two limbs. They are necessarily subject to considerable error, since they involve matters of judgment that cannot accurately be quantitated; but that there are wide, and orderly differences in regenerative activity related to the time of amputation and other factors, is clearly shown in figure 4. From the curves there presented it may be said that, in general, the sooner after irradiation the amputation is performed, the greater is the immediate effect; although the 10-day post-irradiation, light curve might be cited as an exception. As the time between irradiation and amputation increases, however, the immediate delay becomes less, but it may increase progressively so that it appears that limbs amputated early, which were most retarded in the beginning, may turn out better in the long run. The marked effect of photorecovery in the limbs amputated early, as compared to its diminished effect in those amputated late, suggests that although the photochemical part of this process ends at two days post-irradiation when the illumination ceases, recovery of regenerative capacity goes on in the limb for some time after this. Thus, at 10 days, there is still a marked difference in regeneration after amputation between limbs which had been illuminated and those which had not, whereas by 30 days this difference has disappeared. The fluctuations indicated by the 30 day curves in figure 4, probably fall within the error of observation.

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[3] All limbs were not identical in morphological characteristics at the time of amputation, since regression of the digits and forearm was more extensive in some cases than in others, as discussed in previous pages. Figure 2, therefore, is diagrammatic; it shows the level of amputation, but not necessarily the size or characteristics of the digits or other regions of the forearm, which were variable features at the time of amputation.

Obviously the situation is a complex one, and the inherent uncertainty of the comparisons makes it unwise to attempt any detailed analysis in terms of mechanisms. Interesting, nevertheless, is the apparent lack of correlation of the rate of regeneration with the rate of growth of the irradiated limbs, which is seen by comparing figures 3 and 4. Although the minima in both the curves for amputation three days post-irradiation fall within the period of negative growth, the minimum in the dark curve for amputaion, 10 days post-irradiation, falls later (the corresponding light curve shows no minimum). It would seem that the rate of regeneration probably increases with the active growth of the irradiated limb, and that the presence and removal of dead tissue has little or no influence. The general decline in rate of regeneration as the time post-irradiation increases, indicated by the curves for the thirty-day post-irradiation amputation and the 10-day dark curves, remains at the present time without apparent explanation.

A variety of abnormalities may appear in the course of regeneration of the irradiated limb, e.g., partial or complete fusion of digits, reduced number of digits, abnormalities in the size and configuration of digits, presence of supernumerary digits, shortened forearm, etc. Some of these, such as fusion of the digits, are frequently transitory, whereas others are persistent. Figure 5 presents a summary of the distribution of persistent abnormalities in experiment WA as observed at 45 days post-amputation. A high incidence of abnormalities characterizes the irradiated limbs of the animals kept continuously in the dark, whereas those illuminated two days after irradiation show no tendency toward establishment of more abnormalities than do the control limbs. Moreover, the time between irradiation and amputation is without important effect on the incidence of abnormalities. Neither of these findings seems to correlate with the rate of regeneration, where, as we have just seen, both time and photorecovery play a much greater rôle in some cases than in others.

With respect to abnormalities, it should be emphasized especially that here we are concerned with phases of regenerative activity not dealt with at all in previous pages, namely, differentiation and morphogenesis. The inability of a limb to give rise to a regenerate, which is normal in the composition and interrelations of its component elements, rests on some type of interference with the morphogenetic capacities of the cells which were mobilized to establish the blastema. The apparent lack of correlation between the incidence of abnormalities and the rate of regeneration may well mean that by our experimental procedure we have separated (at least partially) the capacity which the cells of the blastema possess for multiplication, from their capacity to differentiate and

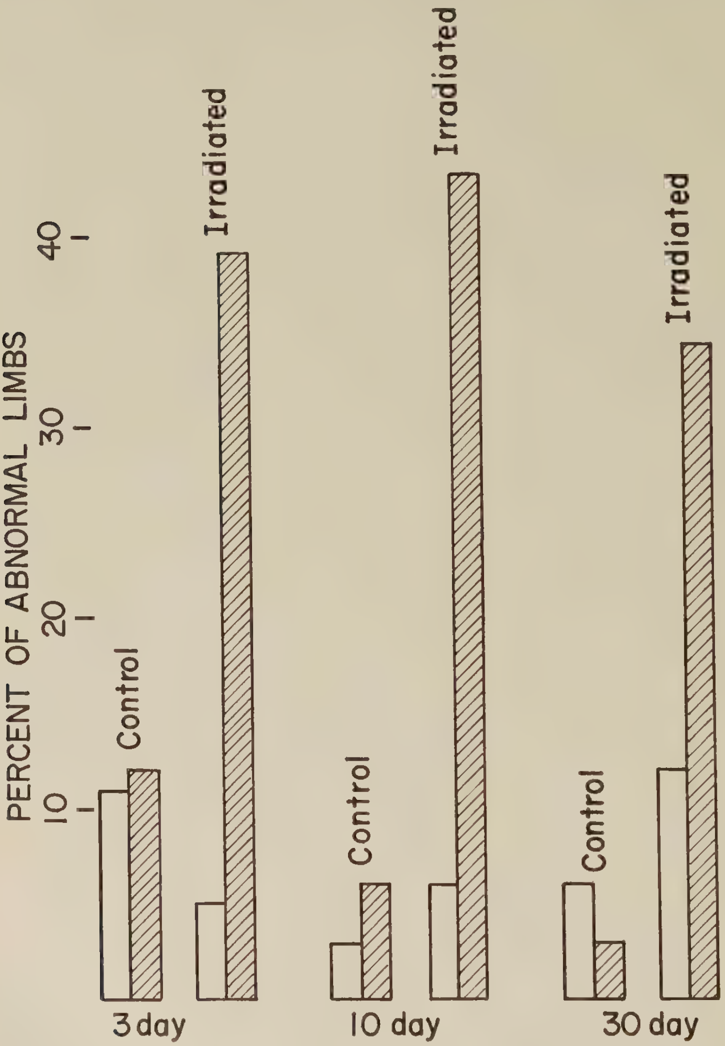

Fig. 5 Comparison of the incidence of abnormalities in forelimbs regenerating after amputation, at 3, 10, and 30 days post-irradiation. Clear rectangles represent animals illuminated for two days after irradiation; shaded rectangles, animals kept in dark after irradiation.

(as aggregations of cells) enter into morphogenetic expression. A comparison may be drawn between the present situation and our previous studies on the development of accessory hands after localized exposure of the limb to ultraviolet light (Butler and Blum, '55). In the latter instance, irradiation of the elbow resulted in the establishment of a supernumerary blastema which underwent growth and morphogenesis. But, among the large number of cases of accessory hands so produced, never have we observed one which was completely normal in the composition and relationship of parts, although some are approximately normal in size. Normal growth in terms of size and rate do not necessarily involve normal differentiation and morphogenesis. Additional experiments are being designed to study further the problems of radiation-induced abnormalities as they occur in the regenerating limb.

SUMMARY

Three effects of localized ultraviolet radiation on the forelimb of larvae of the urodele, *Amblystoma*, have been studied: (1) regression of the irradiated portion of the limb; (2) retardation of regeneration after amputation; (3) production of abnormalities in the regenerate. By application of photorecovery, it has been possible to show that these three effects are essentially separable, although they are probably all expressions of the same kind of photochemical changes.

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THE EFFECTS OF SEVERAL CHELATING AGENTS ON EXCITATION AND CONDUCTION IN FROG SCIATIC NERVE

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THREE FIGURES

Certain compounds used in inorganic analysis form metal-chelates. Some of these compounds exert a powerful binding action on heavy metals and provide a useful tool for the biologist as well as the analyst. If these agents interfere with a biological process, one may hypothesize that a metal is involved.

The possibility that heavy metal-activated reactions are required for nerve excitation and conduction was tested with several chelating agents. The effects resembled those caused either by several conventional inhibitors of carbohydrate metabolism or by procaine, a local anesthetic. There were no clear-cut signs that trace metals participate directly in the processes which were studied. However, there were certain anomalies caused by one compound (cupferron) that indicate the problem may still be open.

METHODS

Sciatic nerves were dissected from *Rana pipiens*. These were used immediately or after an overnight soaking in Ringer's fluid at 4°C. No significant differences were noted as a result of this, except that freshly prepared nerves showed a slow increase of threshold during the first few hours. The desired compounds were dissolved in a Ringer's fluid of the

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following composition: 0.110 M NaCl, 0.0025 M KCl, 0.0013 M $CaCl_2$, 0.0023 M $NaHCO_3$, and 0.001 M phosphate buffer at pH 7.2–7.4. The experiments were performed as follows. A group of nerves were first placed in Ringer's fluid. They were removed and tested periodically during a one to three hour control period. When the nerves gave reproducible readings, some were transferred to the chelater solution. The rest were kept in the Ringer's fluid as controls. The nerves were bathed in the solutions (usually 50 to 100 ml in volume) except for periodic removals to determine threshold and conduction. Although the nerves were placed on the electrodes a number of times, the values of the controls agreed well. The indices chosen for study were: (1) maximum spike potential height, (2) rheobase (measured with a 5 msec. duration pulse), (3) short-shock threshold (25 μsec. pulse), and (4) the magnitude of the stimulus which elicited a spike potential height of $\frac{1}{2}$ the maximum (25 μsec. pulse). The injury (crush) potentials of the sciatic nerve and the sartorius muscle were measured with cotton wick electrodes soaked in Ringer's fluid, calomel cells, and a pH meter which had been converted to a potentiometer. The symbol $t_{1/2}$ indicates the time during block development when the spike potential was lowered to 50% of its initial value. Percentages are reported in terms of the control or in terms of the values determined in the initial equilibration period. The concentrations given in the text refer to the additions made to the stock Ringer's fluid. The time for $t_{1/2}$ and block reported in the tables are averages. The actual times ranged about these averages by a maximum of ± 50%.

RESULTS

Sodium diethyldithiocarbamate (DDC). The effects of this compound on spike height and thresholds are listed in table 1 and illustrated in figure 1. The rates of conduction block development seemed to depend more importantly on the season of the year than on the DDC concentration, as if the site(s) of attack was saturated. The threshold data suggest that the block was not caused by a development of inexcitability.

The *increase* of spike height after exposure to DDC (as in fig. 1) requires comment. DDC is supplied as a sodium salt. It is likely that the increased sodium concentration of DDC solutions as compared to the Ringer's fluid is responsible. In a test experiment, the NaCl concentration of the Ringer's fluid was increased by 10^{-2} M. (No DDC was added.) This solution was applied to several sciatic nerves. During the first 200–250 minutes the spike height gradually rose to

TABLE 1

DIETHYL-DITHIOCARBAMATE CONC. (M/L)	NO. OF NERVES AND MONTH	AVERAGE t ½	AVERAGE TIME FOR BLOCK	EFFECT ON THRESHOLDS	NOTES
		minutes	*minutes*		
10^{-4}	1 (Aug.)	....	....	None observed in 24 hours.	
10^{-3}	4 (Feb.)	> 1000	> 1440	Lowered initially. The thresholds were raised slightly only after the spike height had been reduced. See figure 1.	The spike heights of 17 of these nerves were increased at first. Increases of 3 to 60% were observed.
5×10^{-3}	2 (Mar.)	900	> 1000		
	2 (Jan.)	1600	> 1800		
10^{-2}	2 (Jan.)	1600	> 1800		
	2 (April)	900	1400		
	4 (July)	500	< 1400		
2×10^{-2}	2 (Feb.)	....	....		The spike heights had not begun to fall in 300 minutes when the experiment was terminated.

140% (100% equals the original height.) This resembled the early action of DDC, but the thresholds were not lowered by the NaCl treatment. Both fluids increased the Na^+ concentration gradient across the cell membrane. This in turn increased the spike potential, but the magnitude of this change is in the order of 5% or less for 10^{-2} M extra Na^+. This being much less than the observed increase of 40% in the spike height, we must search elsewhere for the answer. At room temperature the spike potential of the frog sciatic is spread nearly 4.5 cm along the nerve (assuming a duration of 1.5 msec. and a propagation velocity of 30 m per sec. for the major components of the spike). The recording electrodes

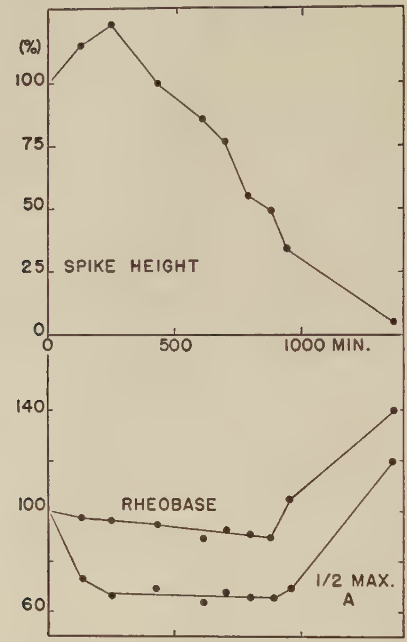

Fig. 1 The action of 10^{-2} M Na-diethyldithiocarbamate on spike height and thresholds (rheobase and 1/2 max. A group). These data were obtained from one sciatic nerve and are fairly typical. The spike height rise at 200 minutes is probably caused by the increased Na ion concentration. Ordinates are in per cent of the readings, obtained during an initial control period when this nerve was in Ringer's fluid.

were separated by only 1 to 1.5 cm., therefore they tended to differentiate the spike. Raising the Na^+ concentration increased the rate of rise of the spike's leading edge. This caused the larger potential difference between the electrodes which was interpreted as a spike increase. Consistently with this explanation, the spike height was initially raised in NaCN and $NaAsO_2$.

The conduction block caused by DDC was reversible. Nerves which had their spike potential lowered to 25% by DDC were placed in Ringer's fluid. Within one hour all measurements returned to their original values, and no further changes were noted during the next 24 hours.

TABLE 2

CUPFERRON CONC. (M/L)	NO. OF NERVES	AVERAGE t ½	AVERAGE TIME FOR BLOCK	EFFECT ON THRESHOLDS
		minutes	*minutes*	
10^{-4}	2	...	....	None observed in 24 hours.
10^{-3}	11	600	1000	In general, half the nerves showed no changes. The others showed a 10–40% drop until block was nearly complete. Just before block the thresholds of all nerves rose not more than 3-fold.
10^{-2}	9	300	500	
2×10^{-2}	8	30	50	

Cupferron (Ammonium nitroso-phenyl-hydroxylamine). NH_4^+ ions can depolarize frog nerve. However, depolarization in 2.2×10^{-2} M NH_4Cl does not begin until 5 hours have elapsed (Gallego and Lorente de Nó, '47). Lower concentrations were reported to have no effect. On this basis, we felt that the ammonium ion of the chelating agent would not interfere if caution was exercised with regard to the concentration and the duration of application.

Table 2 lists the effects on conduction and thresholds of various concentrations of cupferron. In contrast to the action of DDC, cupferron block occurred more rapidly as the concentration was raised and showed no seasonal variation. In almost all experiments, the spike height was depressed in two phases with the suggestion of a plateau between them. What this might represent is not known.

Conduction block was reversible. Nerves fully blocked by 2×10^{-2} cupferron were placed in Ringer's fluid. In only 12 to 15 minutes the spike recovered to 50% of the original height and after 30 to 50 minutes it recovered completely.

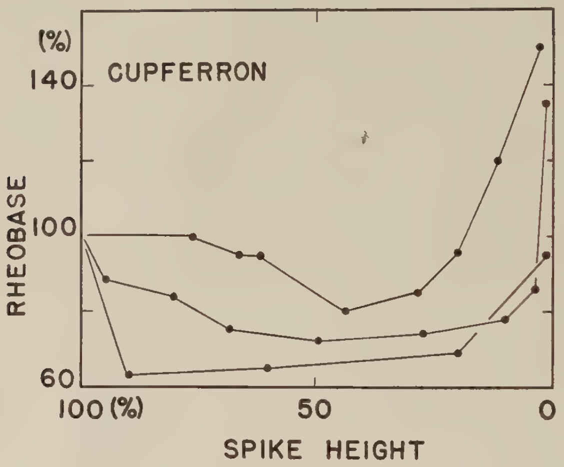

Fig. 2 The rheobases of three nerves exposed to 10^{-2} M cupferron plotted as functions of the spike height at the time of the threshold determination.

Other nerves exposed to lower concentrations were cycled twice through block and recovery. They showed no obvious after effects even though they were tested occasionally for two days following the last recovery. Crab leg nerve (Carcinus) and squid giant axon (Loligo) were also reversibly blocked by cupferron at a concentration of 10^{-2} M.

Depending upon the length of exposure, 10^{-3} M cupferron lowered the injury potentials of 13 sartorius muscles 25 to 75%. Rigor appeared after 16 to 24 hours. Nerves exposed to this concentration were still conducting although their injury potentials were decreased 20 to 50% after 900 minutes. The nerves blocked by 10^{-2} M cupferron were depolarized by only 10 to 30%, whereas several nerves blocked by 2×10^{-2} M were not depolarized. Depolarization and conduction block are apparently unrelated.

Groups of paired nerves were used to assay cholinesterase. One nerve, serving as a control, rested in Ringer's fluid during the time its mate was fully blocked by cupferron (usually 2×10^{-2} M). The nerves were then homogenized separately in 2.0 ml of 0.15 M NaCl, 0.04 M $MgCl_2$, and 0.025 M $NaHCO_3$, and an aliquot of acetylcholine chloride was added (about 1 μM per milligram of nerve). After a one hour incubation period at room temperature, the remaining acetylcholine was assayed with the method of Hestrin ('49). No inhibition of the esterase activity was noted between the blocked nerves and the conducting controls. The average rate was 170 μM acetylcholine per hour-gram wet weight. It does not seem likely that the homogenization procedure diluted the cupferron and reversed a real inhibition because of the small volumes used, the high cupferron concentration, and the fact that no inhibition whatever was found.

The experiments listed in table 3 were performed to test whether or not the NH_4^+ cation of cupferron was as innocuous as initially assumed. NH_4-cupferron was a more potent blocking agent than NH_4Cl (table 3, experiments 1 and 4 versus 2 and 5). There is some sort of synergism between NH_4^+ and cupferron$^-$ as evidenced by a great reduction in blocking time (experiments 4 and 5 versus 6). However, the action of the Na salt of cupferron was surprising. (The Na salt was prepared by passing NH_4-cupferron solutions through a cation exchange resin column.) The blocking effect developed slowly (experiment 3 versus 1) and in addition the thresholds steadily increased as blocking occurred. The plots of threshold against spike height were very similar for Na-cupferron and procaine.

Did the cupferron anion have an anesthetic-like action which was masked by the depolarizing cation NH_4^+? Various concentrations of procaine and NH_4Cl were combined and tested for the purpose of getting a mixture like NH_4-cupferron that would block without raising thresholds. These attempts were unsuccessful. If a NH_4Cl-procaine mixture blocked conduction, the threholds always *increased* as if procaine alone was applied. This threshold effect is manifestly different than that of NH_4-cupferron, so that some action other than anesthesia is indicated for the latter. Certainly, nerves exposed to NH_4-cupferron, are not blocked by an anesthetic-like action because the thresholds are not raised.

TABLE 3

EXP. NO	COMPOUND	CONCENTRATION	ACTION ON SPIKE HEIGHT	ACTION ON THRESHOLDS
1	NH_4-cupferron	2×10^{-2}	t 1/2 was 30 minutes. Conduction blocked in 50 minutes.	Lowered initially.
2	NH_4Cl	2×10^{-2}	The t 1/2 was 500 minutes.	Lowered initially.
3	Na-cupferron	2×10^{-2}	The t 1/2 was 300–400 minutes.	Thresholds increase steadily as in procaine. Block was not relieved by a 3 hour wash in Ringer's fluid.
4	NH_4-cupferron	10^{-2}	Lowered 50% in 240 minutes.	Lowered initially.
5	NH_4Cl	10^{-2}	Lowered 10% in 240 minutes.	Lowered initially.
6	NH_4-cupferron plus NH_4Cl	Both at 10^{-2}	Blocked conduction in 120 minutes.	Lowered initially.

Alpha-furil-dioxime (AFD). This compound blocked conduction fairly quickly but increased the thresholds as the spike height was depressed. This was a constant feature of AFD induced block. Figure 3 compares the action of procaine and AFD with regard to threshold increase as a function of spike height. The similarity leads one to believe that conduction was blocked in both cases by the rising thresholds and not by some specific action on the production of the spike potential by the chelating agent. If AFD blocked nerves were placed in Ringer's fluid only a small spike (ca. 10%) reappeared, and thresholds remained fairly high (two–three times the original values after conduction was restored. If the reversal treatment was applied before conduction was blocked

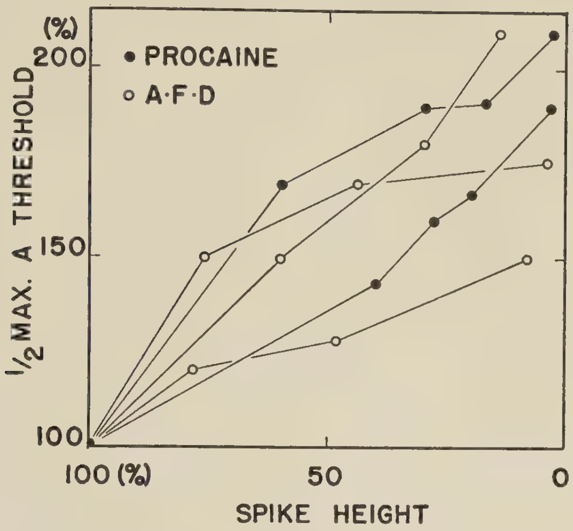

Fig. 3 The effect of 5 × 10^{-2} M alpha-furil-dioxime and procaine on nerve. The shock strength which elicited a response of ½ the height of the maximum A spike is plotted against the maximum A spike height. Time increases along the curves from left to right. The chelater treated nerves took about 200 minutes to block. Procaine blocked in less than 60 minutes.

TABLE 4

ALPHA-FURIL-DIOXIME CONC. (M/L)	NO. OF NERVES	AVERAGE t ½	AVERAGE TIME FOR BLOCK	EFFECTS ON THRESHOLDS
		minutes	*minutes*	
10^{-4}	1	. . .	. . . .	None observed in 24 hours.
2 × 10^{-3}	2	500	1400	Block of conduction appears to be the result of inexcitability. In all nerves, the thresholds were increased as the spike height fell. (Fig. 3.)
5 × 10^{-3}	26	150	200	
10^{-2}	14	60	90	
1.5 × 10^{-2}	3	15	35	

completely, better results were obtained. In several experiments nerves were blocked 50%, were allowed to recover in Ringer's solution, were 50% blocked once again, and were still able to recover their original spike height and threshold in Ringer's fluid.

AFD at 5 and 10×10^{-3} M produced depolarization and rigor in muscle. Muscles were completely depolarized in 240 minutes.

Nerves had injury potentials of 70 to 100% of controls at the time of block (10 nerves in 10^{-2} M). Muscle rigor and nerve conduction block was not prevented by pyruvate, acetate, succinate, or fumarate. These were added to the Ringer's fluid two or 18 hours before the AFD, at concentrations which were double or quadruple the chelater concentration. Succinate may have offered some protection, since the spike took slightly longer than usual to block. The rheobases of these pretreated nerves increased at the usual rate after the AFD was added. The injury potentials were in the same range as in nerves exposed for the same time interval to AFD alone.

The oxygen consumption of 3 separate groups of 8 pooled nerves in glucose-Ringer's fluid was measured in a Warburg apparatus. One group of nerves was then placed in 5×10^{-3} M AFD for 3 hours. During this time 3 of the nerves were completely blocked and the other 5 were 50 to 75% blocked. When the AFD-treated nerves were replaced in the respirometer, they consumed no O_2. The 2 control groups respired at their original rates.

Breis obtained from AFD-blocked nerves split acetylcholine at the same rate as breis from conducting nerves in Ringer's fluid.

Ineffective chelating agents. Several other chelating agents have been tested. Not all of these produced any noticeable effects within 24 hours. This might have been caused by poor penetration or by an inadequate or weak metal binding. The inactive compounds were p-nitrobenzene-azo-alpha-naphthol (saturated solution, ca. 10^{-3}M), anthranilic acid (3×10^{-2} M), and quinaldinic acid (2×10^{-2} M).

Miscellaneous observations. A number of additional experiments were programmed as running comparisons between conventional inhibitors and the chelaters. These compounds probably penetrated the nerve cell at different rates so a comparison of blocking rates was assumed invalid. Instead, the qualitative relation between the spike fall and the threshold alteration was noted.

Sodium iodoacetate (5×10^{-3} M), sodium cyanide (5×10^{-3} M and 2×10^{-2} M), sodium malonate (10^{-2} M), and sodium arsenite (5×10^{-3} M) were tested in the same manner as the chelating agents. Categorically speaking, their action resembled the action of DDC on different time scales (as in fig. 1). Thresholds were slightly lowered as the spike height was gradually reduced. When the spike height reached 10 or 20% of the initial value, the thresholds began increasing. At the time of block the injury potentials were customarily depolarized by 30–40%. These common features are apparently a direct result of the interference with metabolism. Another type of inhibition was tested. The effects of the uncoupling agent, 2–4 dinitrophenol, were studied. This compound (10^{-3} M) blocked nerve in 200–300 minutes. The thresholds were raised slightly (about 50% at $t_{1/2}$), during block development.

Procaine hydrochloride was chosen as a representative anesthetic. This was done to obtain information on how the decreasing spike height during block development was related to the increasing thresholds. Several concentrations were used, but the results will not be retabulated; the effects of procaine are well known (as in fig. 3).

DISCUSSION

Conduction in nerve may fail for several reasons. Among those are: inhibition of metabolism (which supports most cell functions), anesthesia, or interference with the processes which culminate in or constitute an action potential. These possibilities may not be mutually exclusive. This discussion will consider whether or not the effects of the chelating agents are consistent with any of these mechanisms to obtain an "explanation" of their mode of action. It must be confessed that it is not exactly clear to the author what effects would be produced by chelation if metals are directly involved in conduction. Therefore, we cannot conclude that metals are required because we cannot recognize the symptoms of their removal.

TABLE 5

COMPOUND	METALS CHELATED AT NEUTRAL pH
Cupferron	Fe more strongly than Cu.[1] Ca not bound[2]
Na-diethyldithiocarbamate	Zn, Fe, Cd, Co, Pb, Cu more strongly than Mn.[1]
Alpha-furil-dioxime	Co, Cu, Fe^{II} (only), Mn, Zn, Ni.[3]

[1] Martel and Calvin, '52.

[2] Tested by titration.

[3] Personal experiments, chelation assumed if a precipitate appeared upon mixing chelater and metal in solution. No estimate of the chelating power was made.

The metabolic inhibitors caused a fairly consistent series of events. The development of conduction block was initially accompanied by constant or slightly lowered thresholds. This action is characteristic of depolarizing agents (Bishop, '32; Wright, '47). (The rheobase of muscle is lowered as the membrane potential decreases and conduction is blocked when the membrane potential is depolarized to 65% of normal, Jenerick and Gerard, '53.) This pattern of transiently increased excitability, depolarization, and block was also caused by DDC and cupferron, and should lead one to conclude that they are metabolic inhibitors.

Although the chelating agents have an affinity for Fe (table 5), they probably did not inhibit the cytochromes. The Fe of cytochrome is chelated in the porphyrin ring and probably is not free for further chelation. Other metals necessary for glycolysis or in the tricarboxylic acid cycle may have been affected (table 5 is not complete). As far as it has been studied, DDC gave results which are consistent with the action of a metabolic inhibitor. (It inhibits the oxidation of succinate as strongly as cyanide does [Ames, Ziegenhagen and Elvehjem, '46].)

Did cupferron also block conduction by inhibiting metabolism? Probably not. As the concentration of cupferron was increased the spike height fell faster than the injury potential was lowered. Nerves were completely blocked by the highest concentration tested, before any depolarization occurred. Since conduction block without the accompanying depolarization is not generally observed with metabolic inhibitors, some separate action of cupferron is indicated. Winter nerves were not blocked by cyanide until more than 5 hours had elapsed. Cupferron (a much larger molecule) at the same concentration blocked these nerves in less than one hour. Perhaps as a consequence of this there was no marked winter-summer difference in response to cupferron as there was to cyanide and diethyldithiocarbamate. For these reasons, we feel that cupferron's blocking action is independent of its ability to inhibit metabolism.

Is cupferron an anesthetic-like compound? This seems likely for the Na salt of cupferron, but not for the NH_4 salt. Perhaps the cupferron anion is an anesthetic and its ability to raise thresholds was masked by the depolarizing cation NH_4^+. Mixtures of NH_4Cl and procaine like NH_4-cupferron blocked conduction more quickly than NH_4Cl, procaine, or Na-cupferron alone. However, in any mixture of NH_4Cl and procaine yet tried, the thresholds were always raised toward a high value as block developed. In NH_4-cupferron thresholds were not increased until conduction was almost blocked. If an anesthetic is defined as a compound which blocks because it raises thresholds above the safety factor, then we cannot so classify NH_4-cupferron because it does not. Cupferron did not inhibit the enzyme splitting acetylcholine. It may have interfered with the postulated release of this compound, but this was not tested.

Alpha-furil-dioxime, like cupferron blocked faster than cyanide and before any depolarization occurred. However, at any concentration thresholds were always increased before and during the spike fall. Both AFD and procaine blocked in a very similar fashion (see fig. 3). It seems more than likely that AFD block was a consequence of its anesthetic-like action. AFD also inhibited O_2 consumption and depolarized the injury potential but these must have been independent of the blocking action. They followed different time courses than the conduction block.

Crescitelli ('50) has shown that 8-hydroxyquinoline (oxine, a chelating compound) and two chemically related, non-chelating agents block bullfrog nerve in a short time. Thresholds were not reported. Oxine was twice as effective on a molar basis, but different penetration rates may have been involved. Crescitelli's experiments serve to focus attention on the non-chelating portions of these compounds. The AFD molecule consists mainly of hydrophobic groups. These can react with cell lipids and proteins via Van der Waal's forces. The molecule also has a polar-nonpolar configuration which promotes surface accumulation. As Höber ('45) has demonstrated, this set of conditions may allow a molecule to cause narcosis and/or depolarization.

The inhibitor 2–4 dinitrophenol exhibited a weak narcotic action on nerve by slightly raising thresholds, blocking conduction, but not depolarizing. Its structure, with a nitro group ortho to a hydroxyl group suggested to the investigator that it might chelate metals. This notion was tested with the titration method (Martell and Calvin, '52) but no evidence for chelation of Fe or Cu was obtained. The resonance and electron attraction of the nitro group probably prevented the electron donation necessary for chelation.

Although our cholinesterase assays showed no inhibition by cupferron or AFD, other heavy metal chelates are reported to be inhibitory (Dwyer, Gyarfas, Rogers and Koch, '52).

It is impossible to identify the metal ions which were involved. The chelating compounds are not very specific at physiological pH. Assume for the moment that cupferron interferes with conduction by chelating a needed metal-enzyme. Since rather high concentrations of cupferron are needed to cause block before depolarization appears, several conclusions follow. It seems that the chelating agent has a greater affinity for the metabolic catalysts than it has for those which participate in the production of the action potential. If the latter group is more sensitive to metal inhibition, this speaks for the possibility that different metals are involved in the two processes. Alternatively, there may be a preferential accumulation in the neighborhood of the metabolic catalysts.

SUMMARY

1. Frog sciatic nerves and sartorius muscles were exposed to various concentrations of the chelating agents Na-diethyldithiocarbamate, NH_4-nitrosophenylhydroxylamine (cupferron), alpha-furil-dioxime, p-nitrobenzene-azo-alpha-naphthol, anthranilic acid, and quinaldinic acid. The latter three compounds had little or no effect on the spike height, threshold, and injury (crush) potential of nerve.
2. Na-diethyldithiocarbamate slowly blocks nerve conduction without greatly affecting thresholds. It also depolarizes nerve and muscle and causes rigor. Its action is similar to that of cyanide, arsenite, or iodoacetic acid. Like them, it probably acts by inhibiting metabolism.
3. Cupferron also resembles these compounds, blocking and depolarizing both tissues. However, it blocks and depolarizes at different rates; high concentrations can block conduction before any depolarization is found. The cholinesterase of these blocked nerves is apparently not inhibited. Cupferron may interfere with the cellular processes directly underlying spike production, although no direct evidence was obtained.

4. Alpha-furil-dioxime blocks and depolarizes. Thresholds are raised several-fold in nerve as conduction fails. Although block and depolarization go with different time courses, block and increase of threshold tend to follow each other. This compound probably blocks by virtue of an anesthetic action such as is exerted by procaine.

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ACETYLCHOLINE AND EPINEPHRINE EFFECTS ON THE EMBRYONIC RAT HEART[1]

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THREE FIGURES

The effects of acetylcholine and of epinephrine upon the embryonic heart appear to be of special interest, since they are not complicated by possible effects of the drugs on intrinsic nerve cells and fibers and on vessels, as in experimentation on the adult heart.

In the present investigation, acetylcholine (ACh) and epinephrine were tested on isolated embryonic rat hearts and their fragments at stages (10½–13½ days) which precede the invasion of nerve cells and fibers. Additional hearts were used at a stage (14½ days) immediately after such invasion. It was found that both ACh and epinephrine acted directly upon the embryonic rat myocardium.

MATERIALS AND METHODS[2]

The materials and methods were in general those of previous investigations (Hall, '51, '54, '55). Pregnant rats of the Sprague-Dawley strain were anesthetized with sodium barbital (220–300 mg/kg) and the embryos were removed and placed in about 100 cm^3 of a slightly modified Krebs-Ringer solution[3] maintained at 37.5°C. ± 0.7°. The hearts of the embryos were excised and observed under the binocular dissecting microscope.

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In studying ACh and epinephrine effects, two hearts at a time were transferred to a series of 100 cm^3 Krebs-Ringer solutions, in each of which they remained for 5–15 minutes while at least 3–5 determinations of the contraction rate of each heart were obtained (figs. 1, 2 and 3).

The following solutions were used: (a) Krebs-Ringer solution without drugs (initial controls); (b) Krebs-Ringer solution to which ACh had been added just previously; (c) Krebs-Ringer solution to which epinephrine had been added just previously (only one heart of the pair — the more slowly contracting of the two), and Krebs-Ringer solution without epinephrine (the other heart of the pair, for control purposes); and (d) Krebs-Ringer solution without drugs (intermediate control).

This procedure was then repeated and the hearts again transferred to ACh and epinephrine-containing solutions of the same concentrations. This time, the heart that had previously been transferred to the epinephrine solution was used as a control, and the heart that had been used as a control was placed in the epinephrine. Both hearts were finally returned to Krebs-Ringer for terminal control determinations. The entire procedure lasted from 41 to 98 minutes.

The hearts were transferred by pipetting, and were allowed to gravitate into the next solution without transfer of fluid. They could be observed continually while being transferred. Concentrations of acetylcholine bromide ranging from 2.5 to 100 mg/l were used, the cases being distributed with fair uniformity between 5.0 and 80.0 mg/l. Concentrations of epinephrine ranging from 1.25 to 50 mg/l were used, in most cases 5 mg/l of solution.

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[3] Manometric Techniques and Tissue Metabolism, by W. W. Umbreit, R. H. Burris and J. F. Stauffer, Burgess Publishing Co., Minneapolis, p. 119. To this solution was added 0.1% glucose and 0.015% urea. The author is indebted to Dr. William A. Brodsky and Dr. Warren S. Rehm for advice as to the use of this solution.

Average contraction rates for each heart were computed for each stage of experimentation. The 3–5 determinations made during the first 5 minutes in the drug-containing solutions were used in computing the averages, since the effects were more pronounced at this time. Fisher's t values were calculated to determine whether differences between averages were statistically significant. A simplified procedure was used in studying ACh effects on heart fragments.

Thirty 11½ day control hearts were maintained in Krebs-Ringer for 80 minutes; there was no significant difference between the average rate at the beginning (144.0/minute) and at the end (147.7/minute) of this period.

The smallness of these hearts may be emphasized (Burlingame and Long, '39; Hall, '51, figs. 4–10). At 10½ days, the greatest diameter is 0.4 mm; the heart is a U-shaped tube with extremely thin walls. At 11½ days, the diameter is 0.6 mm, yet the thickness of the myocardium does not exceed 45–60 μ. At 13½ days, the heart has acquired its adult conformation, and at 14½ days, its diameter is 2.2 mm. The wall of the atrium is still thin, and the wall of the ventricle highly trabeculated, but the greatest thickness of heart muscle (in the interventricular septum) has attained 0.4 mm.

Nerve fibers do not appear in the heart wall until the 14½ day stage, as demonstrated by silver impregnations (Hall, '51, p. 390). The 10½–13½ day hearts therefore represent stages prior to innervation.

EXPERIMENTAL[4]

ACh and epinephrine effects on the entire heart

A total of 148 hearts were studied and 3,398 determinations of heart rate were made by the standardized procedure

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[4] These results were presented at the Galveston meeting of the American Association of Anatomists, April 1954 (Anat. Rec., *118:* 305–306).

TABLE 1

STAGE	INITIAL CONTROL	ACh	EPI	CONTROL	INTERMEDIATE CONTROL	ACh	EPI	CONTROL	TERMINAL CONTROL
10½	140.4	139.2	172.0	164.1	170.8	167.5	197.5	163.2	167.0
	0.9%		4.8%		1.9%		21.0%		
11½	186.4	142.6	195.2	177.6	188.0	155.6	205.6	162.1	180.0
	23.7%		9.9%		17.2%		26.8%		
12½	200.6	145.9	216.3	184.6	201.3	141.9	215.9	168.8	180.9
	27.3%		17.2%		29.5%		27.9%		
13½	206.2	125.5	198.8	185.3	193.6	128.1	197.2	170.0	165.1
	39.1%		7.3%		33.8%		16.0%		
14½	223.5	94.3	195.1	189.7	184.6	106.1	185.9	156.2	145.1
	57.8%		2.8%		42.5%		19.0%		

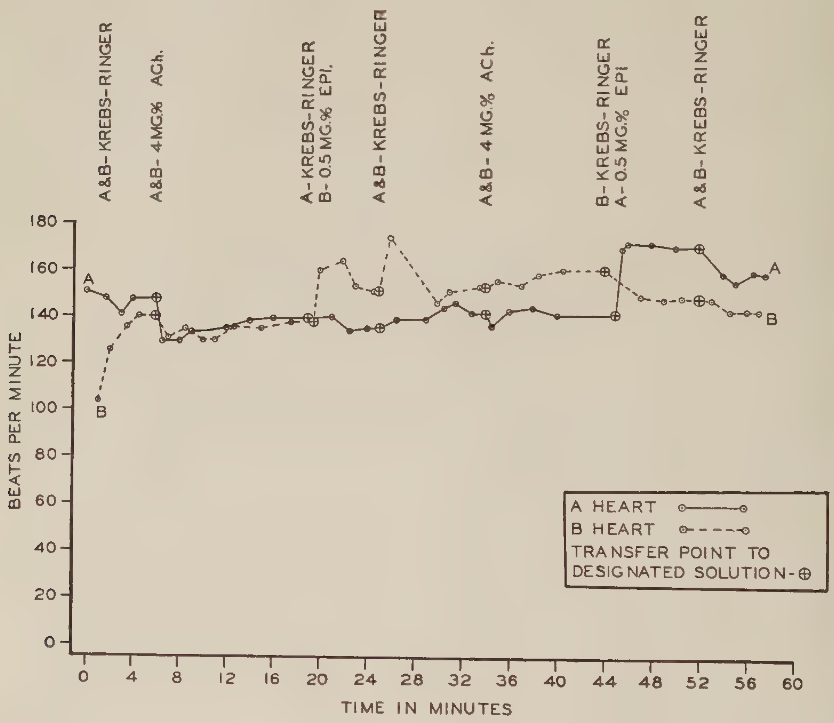

Fig. 1 Graph to show the activity of a typical pair of 10½ day hearts during the standardized procedure. Each circle represents a rate determination obtained by timing 50 beats.

described. Of these hearts, 28 were used at the 10½ day stage, and 30 each at the 11½, 12½, 13½ and 14½ day stages.

Figures 1, 2 and 3 show the activity in beats per minute of a typical pair of hearts of the 10½, the 11½ and the 12½ day stage under the experimental conditions described. Table 1 gives the average contraction rates of the 148 hearts during each experimental period.

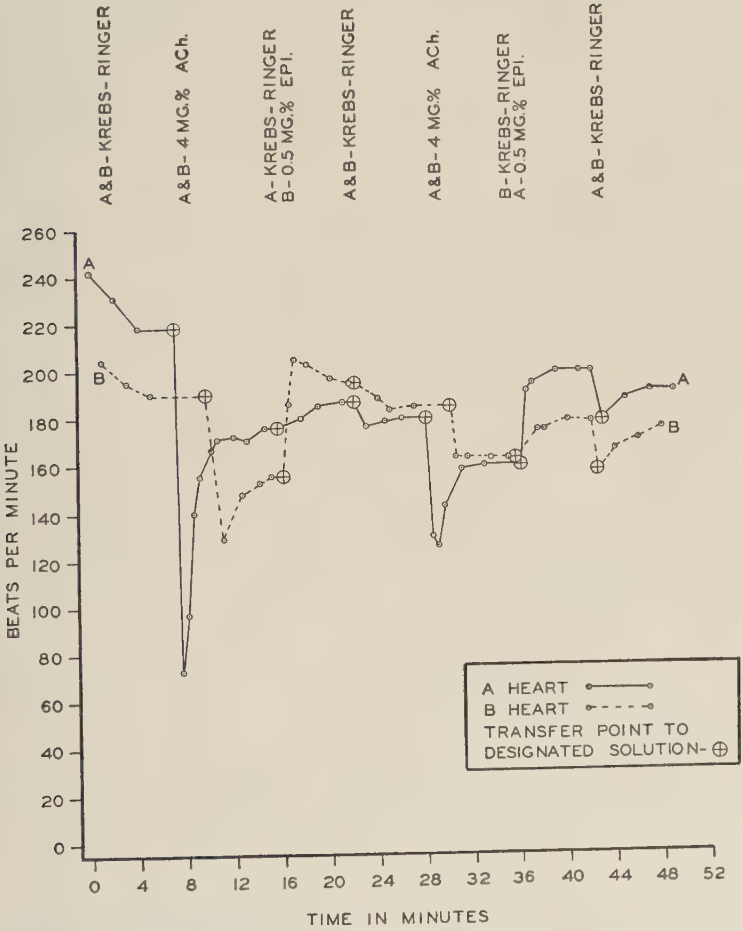

Fig. 2 Graph to show the activity of a typical pair of 11½ day hearts during the standardized procedure.

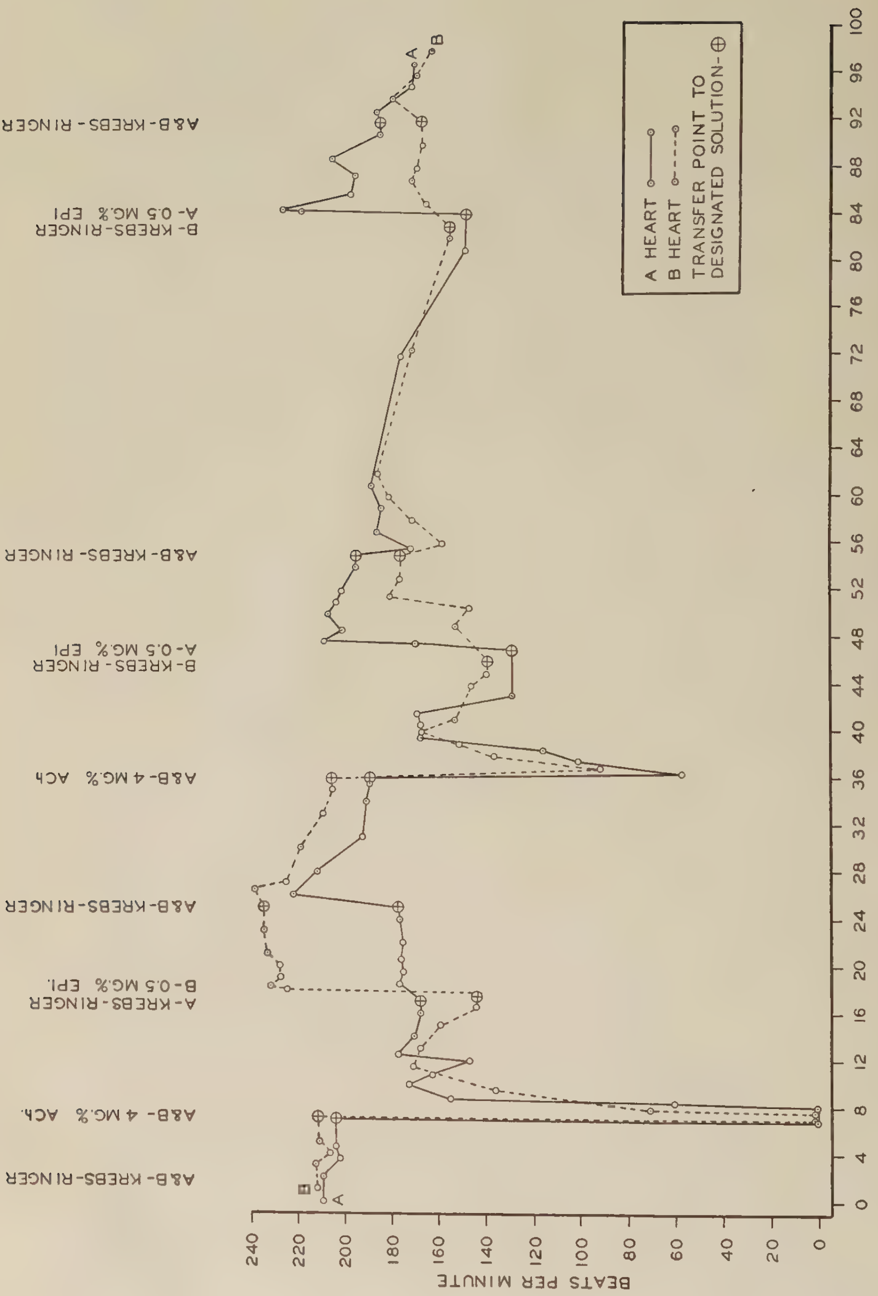

Fig. 3 Graph to show the activity of a typical pair of 12½ day hearts during the standardized procedure, and during a subsequent epinephrine trial.

Acetylcholine effects

There was little variation in the contraction rates of the 28 10½ day hearts. Arrest did not occur in either the first or the second ACh solutions, and the average rates in these solutions were not significantly different from those in the control solutions used just previously.

At the 11½, 12½, 13½ and 14½ day stages, however, both diastolic arrest and deceleration occurred in ACh solutions in many of the 240 trials with these 120 hearts. Arrest was observed in 116 of the trials within one minute after transfer to the ACh solution; 78 arrests occurred during the first ACh trials and 38 during the second. No heart arrested during the second trial which had not already arrested during the first.

The number of arrests tended to increase with increasing age of the heart, from 9 at the 11½ day stage (8 in the first trials and one in the second) to 40 at the 14½ day stage (27 in the first and 13 in the second trials).

The arrest periods lasted only 59.7 seconds on the average, after which the hearts began to beat again, though still in the ACh solution, at rates slower than the preceding control rates. The arrest periods during the first trials were nearly all slightly longer than during the second (averages: 63.3 vs. 52.6 seconds).

Interesting comparisons are possible if the number of arrests at lower dose levels (2.5–10 mg/l) is compared with that at higher levels (40–80 mg/l). In the first trials, more hearts arrested at higher dose levels (80.4% of 46 hearts) than at lower levels (58.3% of 48 hearts). However, in the second trials, more hearts arrested at lower than at higher dose levels (43.7% vs. 23.9%). Hence, the proportion of arrests in both first and second trials was almost the same at lower as at higher dose levels (51.0% vs. 52.2%).

The length of the arrest period seemed not to be correlated with the dose level, for the average arrest time at lower dose levels (in the first trials) is about the same as at higher dose levels (65.3 vs. 61.9 seconds). In a few experiments (not included in the table), even extremely large (640 mg/l and 1,280 mg/l) concentrations of ACh arrested the heart for only a short time (190 and 145 seconds). In only three of the 240 trials did the heart remain arrested during the entire period in ACh solution.

Decelerated activity was also observed in the ACh solutions; this activity followed arrest when such occurred. The average rate reduction augmented regularly in the 60 trials of each of the 11½–14½ day stages, from 20.4% at 11½ days to 50.9% at 14½ days. The reduction in heart rate in the 120 first trials of these stages was greater than that in the 120 second trials (36.9% vs. 30.8%).

At each stage, the reduced average rates were all significantly less than the rates in the control solutions used just before and just afterwards. In only 5 of the 240 trials of these 120 hearts was the average rate in ACh solution faster than the immediately preceding control average.

Epinephrine effects

Significant acceleration was observed in the 14 second trials with epinephrine at the 10½ day stage as compared with the 14 concurrent control hearts, but the slight increase in the 14 first trials at this stage was not statistically significant.

Significant epinephrine effects were also obtained in the 30 first and second trials of the 11½ day series and the 30 first and second trials of the 12½ day series. It will be noted (table 1) that the acceleration was greater in the second than in the first trials.

At the 13½ and 14½ day stages, the average rates in epinephrine solutions were faster than in their concurrent controls. However, even the seemingly large differences in the second trials are significant at only the 5–10% level; the epinephrine effects at these stages are therefore not to be accepted as statistically significant.

ACh effects on heart fragments

Thirty 11½ day hearts were transected at the atrio-ventricular junction, as in a previous investigation (Hall, '51). The initial control average for the 30 sino-atrial fragments was 182.2/minute and for the 30 ventriculo-conus fragments 84.5.

The two fragments were then transferred together to Krebs-Ringer containing either 5.0, 10.0, 20.0, 40.0 or 80.0 mg/l of ACh (6 hearts at each dose level), where they remained for 3–6 minutes.

Nine of the 30 sino-atrial fragments arrested for an average of 55.8 seconds. Fifteen of the 30 ventriculo-conus fragments arrested for an average of 94.2 seconds. During activity, which followed arrest if it occurred, the average sino-atrial rate was 140.2/minute and the average ventriculo-conus rate 64.9, a statistically significant deceleration of 23.1% and 23.2% respectively from the initial control rates.

The fragments were then transferred to terminal control solutions for 3–5 minutes, where the sino-atrial average increased to 155.6/minute and the ventriculo-conus average to 74.3. These rates were significantly higher than those in ACh solutions, but still significantly lower than the initial control averages.

DISCUSSION

According to older concepts, the site of action of the sympatho- and parasympathomimetic drugs is on nerve endings (reviews, Dixon and Ransom, '12; Henderson and Roepke, '37). These concepts were based on the similarity of the drug effects to the effects of electrical stimulation of the appropriate nerves.

Henderson and Roepke ('37), however, concluded for ACh that "It is of course still possible to suggest that the reaction occurs through the hypothetical myoneural junction, but this seems entirely unjustified. We may then more simply think of acetylcholine, set free by nervous impulses or applied, acting upon the cell, producing certain chemical and physical results.''

This conclusion has been supported by the work of a number of investigators on the embryonic chick heart. Plattner and Hou ('31) and Cullis and Lucas ('36) studied the effects of ACh, whereas Fujii ('27), Markowitz ('31), Hsu ('33), and Fingl, Woodbury and Hecht ('52) used both ACh and epinephrine. Barry ('50) studied the effects of epinephrine. All of these authors obtained positive results on the embryonic chick heart at stages prior to innervation, though Fujii ('27) was inclined to relate his results to the innervation of the heart.

Garrey ('37), however, found that chick heart muscle developing in tissue cultures was not inhibited by ACh in any physiological concentrations, and Nordmann and Rüther ('31), working with similar preparations at older (innervated) stages, failed to find stimulating effects with epinephrine, though Varga ('31) reported positive results with such preparations. Armstrong ('35), working on the embryonic fish heart, concluded that ACh effects are mediated only through nerves, and that ACh does not act directly on heart muscle.

Divergent points of view have also been expressed as to the response of adult ventricular muscle to ACh. Hoffman and Suckling ('53) found that vagal stimulation and ACh greatly increased the rate of repolarization in the muscle of the dog atrium, but produced no such change in ventricular muscle. They concluded that ''The lack of sensitivity of ventricular tissue to acetylcholine is most likely due to the fact that cholinergic endings are not present in the dog ventricle . . .'' On the other hand, Lissák and Martin ('40) reported that in Rana esculenta the response of ventricular muscle to ACh (1–10 mg/l) was almost as great as that of the atrium.

The present investigation extends to an embryonic mammalian myocardium the results obtained with ACh and with epinephrine by most investigators on the embryonic chick heart. Embryonic rat hearts responded to ACh at stages prior to innervation (11½–13½ days), though not at the earliest stage used (10½ days). Similarly, positive effects of epinephrine were demonstrated at stages prior to innervation (10½–12½ day stages). These responses were found to be significant by statistical methods on adequate samples.

The response to ACh was more marked in first trials than in second trials with respect to number of arrests and degree of deceleration. The response to ACh of older embryonic hearts was greater than that of younger hearts with respect to the same criteria; this recalls the greater sensitivity of older embryonic chick hearts to ACh and epinephrine found by Fujii ('27) and by Markowitz ('31). The ventriculo-conus fragments of the hearts responded as well to ACh as the sino-atrial fragments.

The dose levels used of both ACh and epinephrine were high as compared with those usually used on the adult heart, but were comparable to those used on the embryonic chick heart. Nearly all of the embryonic rat hearts recovered from arrest while still in the ACh solution; such recovery could not have been due to the action of cholinesterase, or to the liberation of an epinephrine-like substance, as suggested for the adult heart (Hoffmann, Hoffmann, Middleton and Talesnik, '45).

The experiments with the 11½ day rat hearts may be considered the most satisfactory, since the intermediate control average and the terminal control average do not differ significantly from the initial control average.

The terminal control averages of the 13½ and 14½ day hearts, however, are significantly lower than the initial control averages, and thus progressive deceleration may account in part for the rate reduction in ACh solutions at these later stages. Similar considerations apply to the second ACh trials of the 12½ day stage. Nevertheless, the deceleration in ACh solutions greatly exceeds that in the subsequent control solutions.

An increase of the intermediate and terminal control averages of the 10½ day hearts as compared with the initial control average will be noted. The lower initial control rate may perhaps be attributed to relatively greater trauma during excision of this very small heart. The much larger hearts of the 13½–14½ day stages, on the other hand, were probably much less traumatized during excision; their large size, however, probably accounts for their failure to accelerate significantly in epinephrine solutions. This is in accord with Hsu's ('33) experience with the embryonic chick heart.

Barry ('50) found that the accelerating effects of epinephrine were greater the more the embryonic chick hearts were depleted. The present observations support this finding, since the second epinephrine trials resulted in greater acceleration than the first trials.

For ACh, it may be pointed out that a growing body of evidence indicates that this substance is not merely concerned with the transmission of nervous impulses, but is involved in the contraction mechanism as well (reviews, Burn, '50; Rothschuh, '54). The present findings are consonant with this revised concept of the role of ACh.

SUMMARY

This investigation dealt with the reactions of 148 embryonic rat hearts of the 10½–14½ day stages to acetylcholine (2.5–100 mg/l and to epinephrine (1.25–50 mg/l) dissolved in Krebs-Ringer solutions in which the isolated hearts were maintained at 37.5°.

Hearts of the 10½ day stage failed to respond to ACh. Hearts of the 11½–14½ day stages, however, responded by temporary diastolic arrest (average: 59.7 seconds) in 116 of 240 trials; such arrests were more numerous in first than in second trials of the same heart at the same dose level (78 vs. 38).

Whether temporarily arrested or not, 11½–14½ day hearts beat more slowly in ACh. The average rate was reduced 36.9% in first trials and 30.8% in second trials of the same hearts.

The average deceleration augmented from the 11½ to the 14½ day stage (20.4%–50.1%). Arrests increased from 9 to 39 in the 60 trials of the 30 hearts at each stage.

Ventricular fragments of the 11½ day heart responded as well to ACh as sino-atrial fragments.

Epinephrine effects were demonstrated by transferring one heart of a pair from ACh solution to epinephrine-containing Krebs-Ringer, the other heart to Krebs-Ringer (control). There was significant acceleration in epinephrine in the second trials of the 10½ day stage, and in first and second trials of the 11½ and 12½ day stages.

The experiments thus demonstrated ACh effects before innervation (11½–13½ day hearts) and immediately after innervation (14½ day hearts). The experiments also demonstrated epinephrine effects at stages before innervation.

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THE METABOLISM OF ADIPOSE TISSUE UTILIZING NEOTETRAZOLIUM

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ONE FIGURE

Although evidence for the metabolic versatility of adipose tissue exists (Wertheimer and Shapiro, '48; Editorial, '50; Bremer, '38; Shapiro and Wertheimer, '43; Mirski, '42; Stern and Shapiro, '54), detailed information concerning specific enzymatic mechanisms involved is incomplete. This may be due to the difficulties encountered in the application of classical biochemical techniques to a study of the quantitative metabolism of adipose tissue because of (a) extreme variations in lipid content and even fat-free dry weight of this tissue (Hook and Barron, '41), and (b) the obstacles involved in the cutting of uniform thin slices of fresh tissue. The tetrazolium salts, which have been shown to provide a convenient parameter of enzymatic activity in a variety of tissues (Antopol et al., '48, '50; Shelton and Schneider, '52; Bourne, '53), afford an alternative approach to the delineation of the enzymatic characteristics of adipose structures. In the present investigations the enzyme systems of human adipose tissue have been studied with various substrates utilizing the reduction of neotetrazolium (NT) to its insoluble formazan as an indicator of activity.

MATERIALS AND METHODS

Pieces of fat of approximately 0.5 cm thickness were sliced freehand from fresh surgical specimens of perirenal fat, omentum, or breast and placed in 5 ml Stender dishes. The incubation medium consisted of 5 ml of 0.5% NT in phosphate-buffered Ringers (Fried and Zweifach, '55). The substrates (Na salt) alpha-glycerophosphate (AGP), succinate, lactate, glucose, and mannose were used at a final concentration of 0.5%. Coenzyme I (DPN) was always added in final concentrations of 0.01% to the solutions containing AGP and lactate. To test for the presence of endogenous substrates, controls without added substrate were run both with and without DPN. The effect of malonate, monoiodoacetic acid, and fluoride on the reduction of NT in the presence of each of the above substrates was also determined. In order to test the effect of freezing on biochemical viability, several samples of fat tissue were frozen for 24 hour periods and then assayed for enzymatic activity.

The samples were incubated at 37°C. in an air oven for 24 hours, since shorter incubation periods did not assure maximal reduction of NT. Following incubation, the intensity of staining of the lower surface of each slice of fat tissue was evaluated in terms of slight, moderate, or marked. Frozen sections of representative samples were prepared for microscopic examination.

RESULTS

Reduction of NT by fat tissue without added substrate was absent or extremely slight, indicating negligible endogenous activity; in the presence of DPN endogenous reduction resulted in atypical bright red staining rather than the usual purple. Most intense reduction of NT occurred consistently with AGP (DPN) as a substrate; moderate reduction took place with succinate and lactate (DPN), while with mannose and glucose there was only slight formazan formation (table 1). Omitting DPN with either AGP or lactate resulted in an appreciable diminution of tetrazolium reduction. The addition of DPN to tissue incubated with succinate produced no observable increase in formazan.

The action of specific metabolic inhibitors is summarized in table 2. Concentrations of inhibitor below the cited levels were ineffective.

The effect of freezing on specific enzymatic activity was determined in tissue blocks frozen for 24 hours. Complete loss of tetrazolium reducing activity was encountered with glucose and mannose, slight decreases for lactate and succinate, while there was no observable decrement with AGP.

TABLE 1

NT reduction by adipose tissue with various substrates

SUBSTRATE	NO. OF DETERMINATIONS	INTENSITY OF NT REDUCTION
Endogenous control	7	Negligible
Endogenous + DPN	12	Atypical
Mannose	9	Slight
Glucose	5	Slight
Succinate	15	Moderate
AGP[1] (DPN)	18	Marked
Lactate (DPN)	7	Moderate

[1] Alpha-glycerophosphate (Na).

TABLE 2

Inhibition of NT reduction in adipose tissue by metabolic poisons

SUBSTRATE	FLUORIDE (0.01 M)	MALONATE (0.03 M)	MONOIODOACETIC ACID (0.005 M)
Glucose	Marked	None	Moderate
Mannose	Marked	None	Moderate
AGP (DPN)	None	None	Marked
Succinate	None	Marked	None

Microscopic examination of frozen sections of treated adipose tissue showed deposition of formazan granules only in the cytoplasmic rim of the fat cell (fig. 1), which is in agreement with a previous study on the localization of succinic dehydrogenase in adipose tissue (Padykula, '52). The lipid substance proper usually stained a diffuse pink, with discrete spicules of formazan occasionally observed. Histological differences in the degree of formazan deposition with different substrates could not be evaluated because of the heavy density of granules (fig. 1).

DISCUSSION

NT has been used with a variety of substrates and cofactors to indicate the presence of the following enzyme systems in adipose tissue: alpha-glycerophosphate dehydrogenase (DPN), succinic dehydrogenase, lactic dehydrogenase, and enzymes of the glycolytic cycle. Highest activity was observed for AGP-dehydrogenase, next in order for succinic dehydrogenase and lactic dehydrogenase, and lowest in glyco-

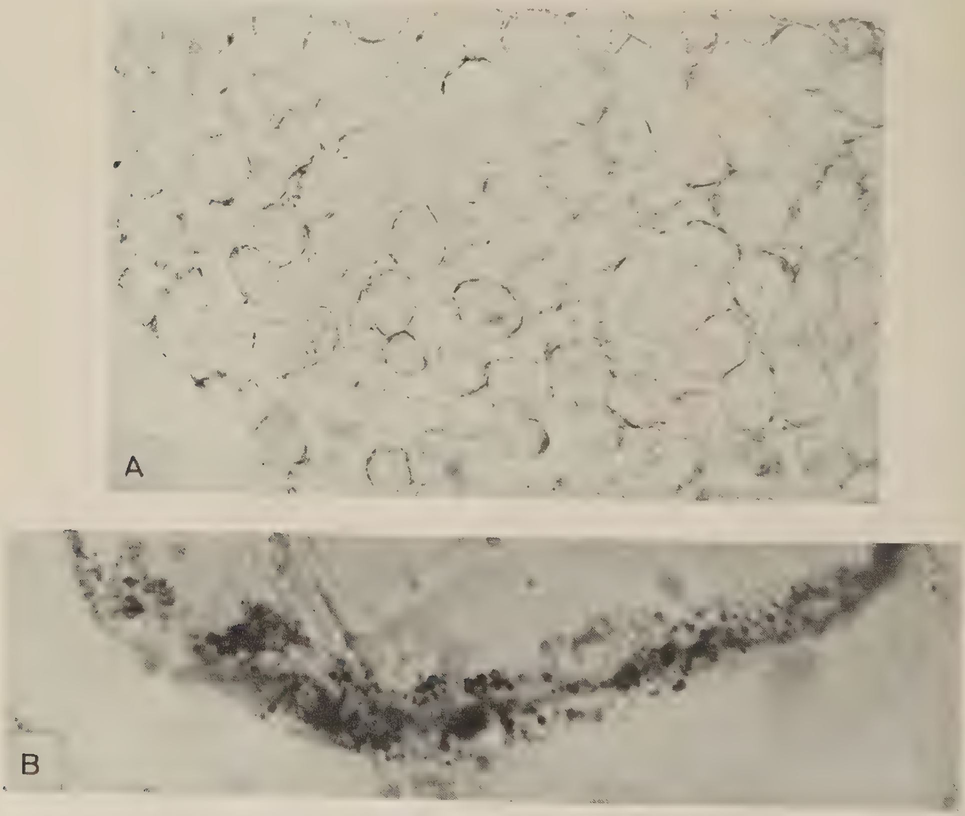

Fig. 1 Formazan deposition in cytoplasmic rim of the fat cell.
A. magnification × 100. B. magnification × 1200.

lysis. Since the addition of DPN to fat tissue incubated in succinate produced no observable increment in formazan, the intensity of staining obtained with AGP (DPN) and lactate (DPN) is felt to reflect the actual activity of the associated enzyme systems rather than an additive effect of enzyme and coenzyme acting independently. The bright red staining obtained with DPN in endogenous samples probably indicates a weak reduction of the ditetrazolium to a monoformazan.

In an earlier study utilizing the Thunberg methylene blue technique (Shapiro and Wertheimer, '43), more intense oxidation by adipose tissue extracts was reported with succinate than with AGP. Strict comparison of these findings with those of the present investigation is not possible however, because these investigators did not utilize DPN in measuring the oxidation of the triose. Padykula ('52) has more recently demonstrated the existence of succinic dehydrogenase in the cytoplasm of adipose cells with a similar histochemical technique. Since AGP-dehydrogenase serves to link the metabolic pathways of fat and carbohydrate its high activity in adipose tissue supports the concept of active metabolic interconversion in this tissue (Tuerkischer and Wertheimer, '42).

The validity of the technique employed as a parameter of enzyme activity was fortified by the inhibitory effect observed with the metabolic poisons utilized here. Thus, monoiodoacetic acid, chiefly an inhibitor of triose oxidation, exerted a marked inhibitory effect on NT reduction with the triose AGP, moderate inhibition with glucose and mannose, and no effect with succinate. Malonate suppressed reduction only with succinate as a substrate, indicating the involvement of the succinic dehydrogenase system. The action of fluoride, a general inhibitor of glycolysis, suggests that the oxidation of glucose and mannose by adipose tissue involves a fermentation rather than a direct dehydrogenation (Antopol et al., '55).

It is of interest that the changes involved in freezing impaired NT reduction most markedly with the hexose sugars as substrates, possibly indicating a dependency upon the integrity of intra-cellular structures of the enzymatic mechanisms involved in glycolysis.

A number of investigators have noted the staining of fat depots in the proximity of actively metabolizing structures following incubation of tissue in tetrazolium (Zweifach et al., '51). The present study indicates that the granular staining of the cytoplasm proper of the fat cell represents indigenous enzymatic activity rather than a migration of formazan from sites of active reduction in surrounding tissues. This is not to be confused with the diffuse staining of the fat globule within the cell, which may be due to migration of formazan from the surrounding cytoplasm into the lipid moiety. This possibility of even limited shifts in formazan configurations emphasizes the hazard of drawing too rigid a conclusion concerning the significance of the patterns of intra-cellular formazan in enzyme topography (Weibull, '53).

SUMMARY

Neotetrazolium (NT) has been used for histochemical study of enzymatic activity in adipose tissue. With alpha-glycerophosphate (DPN), NT reduction was most intense, moderate reduction was observed with succinate and lactate (DPN), while with glucose and mannose there was only slight formazan formation. Endogenous activity was negligible.

The inhibition of NT reduction by monoiodoacetic acid, malonate, and fluoride indicated that tissue enzyme systems were involved.

Tissue samples frozen for 24 hours prior to incubation showed marked impairment of NT reduction with glucose and mannose, moderate decreases with lactate and succinate, and no loss in activity with alpha-glycerophosphate.

It is suggested that formazan in the cytoplasm of the adipose cell arises from indigenous enzymatic activity.

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THE LUMINESCENT REACTION IN EXTRACTS OF THE MARINE DINOFLAGELLATE, GONYAULAX POLYEDRA[1]

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NINE FIGURES

INTRODUCTION

Bioluminescence, or the production of light by living organisms, occurs in a great variety of unrelated and diverse animals and plants (Harvey, '52). The nature of the luminescent display may vary, but the reaction in all cases is evidently an enzymatic chemiluminescence, usually an oxidation. The bioluminescent reaction has been carefully studied in only three groups of organisms, namely *Cypridina*, fireflies and bacteria. The light emitting reaction of each differs markedly in requirements (Tsuji, Chase and Harvey, '55; McElroy and Hastings, '55; Strehler, '55; Hastings and McElroy, '55). The present paper, which describes characteristics of the reaction in *Gonyaulax*, indicates that this reaction differs from the three previously described.

Gonyaulax polyedra is a marine armored dinoflagellate which is both photosynthetic and luminescent. The so-called phosphorescence or, more properly, luminescence of the ocean at night is generally believed to be due principally to the luminescence of dinoflagellates (Harvey, '52). *Gonyaulax* has recently been isolated and grown in pure culture by one of the authors (B.M.S.), making possible a study of its physiology and biochemistry. The characteristic luminescence of *Gonyaulax* occurs upon stimulation as a brilliant flash, having a duration of about 0.1 sec. (fig. 9). Maximum luminescent responses occur in darkness and are about 40 to 60 times as bright as the responses in daylight. A preliminary account of the luminescence of living *Gonyaulax* has been given by Haxo and Sweeney ('55) and more detailed studies along these lines are published elsewhere (Sweeney and Hastings, '57).

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[1] This research has been supported in part by grants from The National Science Foundation and the Graduate School of Northwestern University. Contribution from the Scripps Institution of Oceanography, University of California, New Series No. 912.

MATERIALS AND METHODS

Cells were grown in Fernbach flasks, each containing 1500 ml of medium, placed on shelves in windows (north exposure) at room temperature (23°–26°C.). The culture medium was composed of 75% filtered sea water, 2% soil extract (Pringsheim, '46), 2×10^{-3} M KNO_3, 2×10^{-4} M K_2HPO_4, 6×10^{-6} M $FeCl_3$, and 2.7×10^{-5} M ethylenediaminetetraacetic acid (EDTA) at pH 8.0–8.2. All distilled water used in these experiments was distilled a second time in a glass still.

Cells were inoculated at a cell density of about 800 cells per milliliter and harvested 6 days later at a density of about 6000 cells per milliliter. In preliminary studies it was found that extracts from cells harvested at midnight were about 10 times more active than those from cells harvested at noon, so the procedure was carried out thereafter at midnight. The cells were harvested with a Buchner funnel on Wratten no. 4 filter paper, washed several times with distilled water to remove residual salts and plunged into cold acetone at —25°C., removing a considerable amount of the pigment. The acetone was removed by filtration and the resultant powder dried *in vacuo* over calcium chloride and paraffin. A yield of about 45 mg of dry powder per liter of culture was generally obtained. Light intensity was measured with highly sensitive apparatus, utilizing a photomultiplier tube as described previously (Hastings, McElroy and Coulombre, '53), and recorded on an Esterline-Angus graphic meter.

EXPERIMENTAL RESULTS

Crude extracts

When initial preparations of the dry powder were mixed with distilled water a very faint, slowly decaying luminescence was observed. However, when distilled water was replaced with sea water, the intensity was increased several fold, suggesting a salt requirement in the reaction. In subsequent preparations care was taken to remove residual salt by washing the cells with distilled water, as described above. Such salt free powders then gave essentially no light when mixed with distilled water, but when optimum salt was added, a dim, but readily visible, luminescence occurred. The salt-free powder therefore was routinely extracted with distilled water (20 mg/ml) in the cold with a Potter homogenizer and centrifuged for 10 min. at 20,000 RPM in a Spinco preparative centrifuge (head no. 40) for 10 minutes at 0°C. The clear yellow-green supernatant contained the activity.

It was found that NaCl would completely replace sea water in stimulating luminescence. As shown in figure 1, unusually high concentrations of salt, about 1.2 M, are required for maximum activity. It was found that KCl, $(NH_4)_2SO_4$, and sodium and potassium phosphates (pH 6.6) were equally effective at the same concentration in replacing NaCl for the stimulation of luminescence. Versene (EDTA) was not inhibitory in a final concentration of 10^{-3} M when added secondarily. The salt requirement was also found in partially purified preparations of the enzyme (see below). The role of salt in this reaction is not clear and has not been studied in any detail. Salt in lower concentrations is known to affect light production in other luminescent reactions however (Anderson, '37; McElroy, Hastings, Sonnenfeld and Coulombre, '54).

The reaction is markedly dependent upon pH, as shown in figure 2, with an optimum at about pH 6.6. Similar results have been obtained using sodium phosphate and potassium phosphate buffers, including the sharp drop in activity above

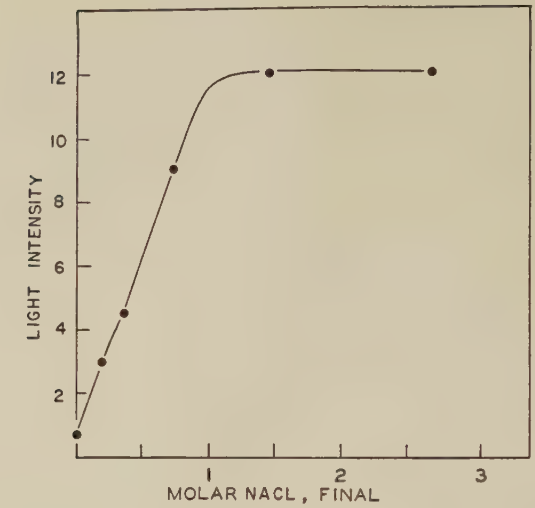

Fig. 1 Relationship between NaCl concentration and light intensity. Reaction mixture contained 1.0 ml crude extract, 0.4 ml 0.05 M tris-maleate buffer pH 6.4, water and NaCl to a total volume of 2.5 ml.

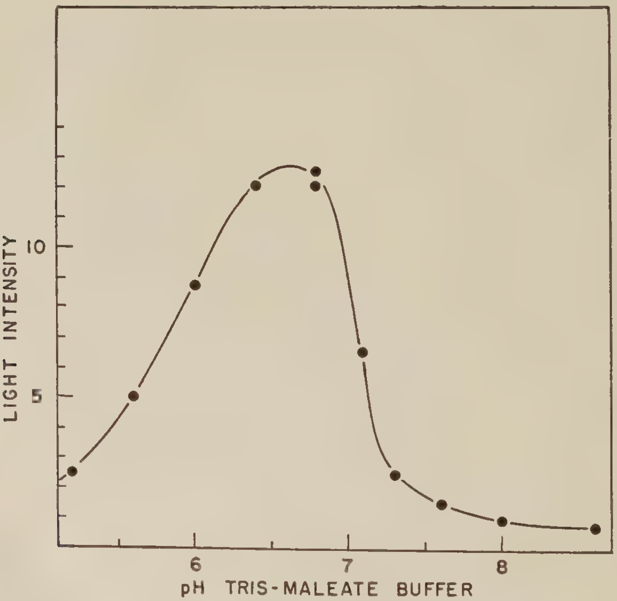

Fig. 2 Relationship between pH and light intensity. Reaction mixture contained 1.0 ml crude extract, 0.4 ml 0.05 M tris-maleate buffer, 0.6 ml 6.1 M NaCl, and 0.5 ml H_2O.

pH 7. There was little difference betwen different buffers in the maximum luminescence obtained, and tris-maleic acid buffer (trihydroxyamino-methane and maleic acid adjusted to pH with NaOH) was used routinely in all experiments.

The light emitted by the living cells is blue to blue-green. Utilizing concentrated extracts, it has been possible to determine the emission spectrum of the *in vitro* reaction. As shown in figure 3, the emission peak is at about 478 mμ. Since ammonium sulfate in place of NaCl had been found to result in a much slower decay of luminescence, it was used in these experiments. The reaction mixture was placed at the entrance slit of a Bausch and Lomb grating monochromator, and the photomultiplier tube at the exit slit. Both slits were set at 5 mm and the light intensity was recorded at various wave length settings. The values obtained were corrected both for

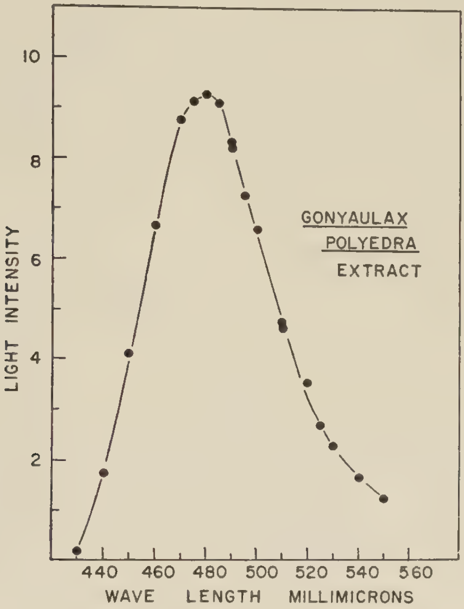

Fig. 3 Emission spectrum of *Gonyaulax* extracts. Reaction mixture contained 1.6 ml crude extract, 0.2 ml 0.1 M tris-maleate buffer pH 6.6, and 0.2 ml 6 M $(NH_4)_2SO_4$. Time during determinations, 6 min.

the decay of the reaction and for the color sensitivity of the phototube.[2] A second determination gave identical results.

The effect of temperature upon the reaction was determined both with the crude extract and with the purified enzyme (see below). In both cases the optimum was found to be at about 24°C., and a curve for the purified enzyme is shown in figure 4. The reaction mixture without enzyme was adjusted to the desired temperature and placed in front of the phototube. Enzyme was rapidly added to initiate the reaction and the initial light intensity recorded as the reaction rate. An Arrhenius plot of the data indicates an activation energy of about 9000 calories, and a similar plot for the rate of enzyme inactivation by heat gives an activation energy of about 60,000 calories.

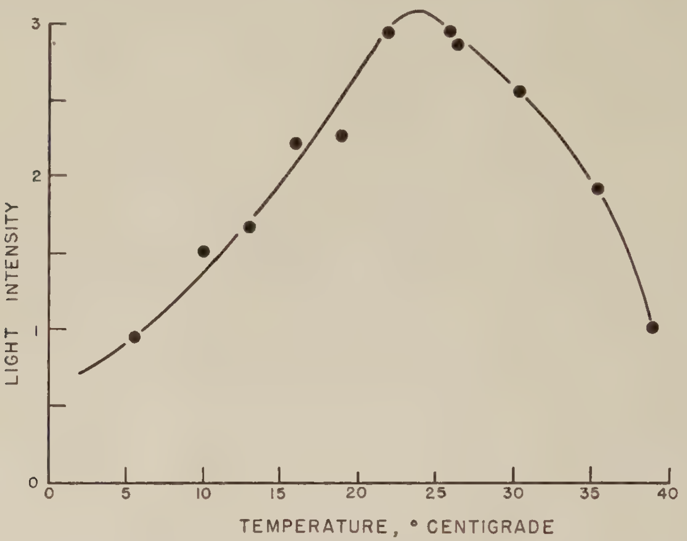

Fig. 4 Effect of temperature upon luminescence. Reaction mixture contained 0.2 ml purified enzyme, 1.0 ml boiled crude extract, 1.2 ml 6.1 M NaCl, 0.4 ml 0.1 M tris-maleate buffer pH 6.6, and 1.2 ml H_2O.

Enzymatic activity was completely destroyed at 100°C. for one minute, but the loss of activity of purified enzyme was less than 5% after two hours at 22°C. However, the crude extract is quite unstable and loses activity relatively rapidly, as shown in figure 5. In this experiment the crude extract was kept in an ice bath at about 4°C. and at the times indicated was assayed for activity. Varying the temperature of the extract from 4° to 30°C. during this period had only a relatively small effect upon the rate of decay. This suggests that the decrease is not due to enzyme destruction since denaturation is generally characterized by a large temperature coefficient. Additional evidence for this is presented in a later section. The possibility that a slow auto-oxidation of substrate was taking place was tested by bubbling aliquots with pure oxygen and pure nitrogen. In each of three trials oxygen not only failed to accelerate the decay of the extract but actually resulted in a slight increase in activity. It is considered possible that the decay is due to the production of potent in-

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[2] We are grateful to Mr. John Tyler for determining the color sensitivity of our phototube.

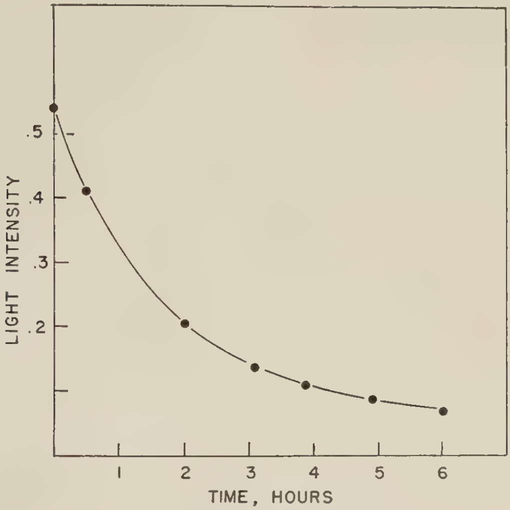

Fig. 5 Spontaneous decay in activity of crude extract kept at 4°C. Aliquots were taken at time intervals indicated and assayed for luminescence. Reaction mixture contained 0.5 ml crude extract, 0.4 ml 0.05 M tris-maleate buffer pH 6.6, 0.6 ml 6.1 M NaCl, and 0.5 ml H_2O.

hibitors since old extracts which have decayed are highly inhibitory to fresh extracts.

The effect of concentration of crude extract is shown in figure 6. The shape of the curve indicates that a second factor, presumably substrate, is limiting in the reaction at low concentrations.

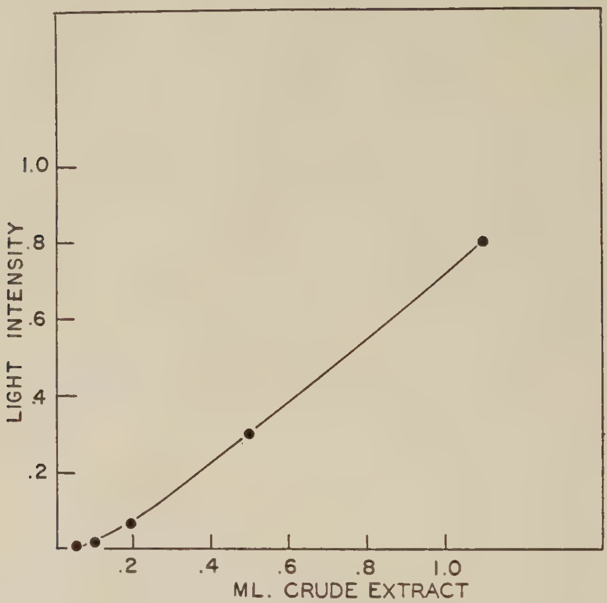

Fig. 6 Relationship between the concentration of crude extract and light intensity. Reaction mixture consisted of 0.5 ml 6.1 M NaCl, 0.4 ml 0.05 M tris-maleate buffer pH 6.6, and water and enzyme to a volume of 2.0 ml.

The intensity of luminescence of the crude extract is dependent upon oxygen concentration. When hydrogen is bubbled through a luminescing reaction mixture, the intensity decreases to a low level. When air is subsequently readmitted, the intensity is increased. No excess flash of luminescence, such as has been reported in the case of extracts of *Cypridina*, (Chance, Harvey, Johnson and Millikan, '40), the firefly (Hastings, McElroy and Coulombre, '53) and the luminous bacteria (Hastings, '55), was observed. It may be concluded however that the *Gonyaulax* reaction is an oxidative one similar, in this respect, to other bioluminescent reactions.

Purified enzyme

Preliminary purification procedures have resulted in the separation of the enzyme from the heat stable substrate or substrates. Ammonium sulfate was added to the crude extract to 35% saturation, and allowed to stand for 15 minutes at 5°C., and centrifuged at 25,000 RPM (Spinco, Head no. 40) for 12 minutes. A green precipitate resulted, which was inactive, insoluble in water, methanol soluble, and showed absorption suggesting a mixture of chlorophyll and carotenoid pigments. Ammonium sulfate was added to the supernatant to 75% saturation and the resulting precipitate was clear and readily soluble in water. By itself this fraction was completely inactive, but when added together with a boiled crude extract, excellent activity resulted. Figure 7 shows the effect of purified

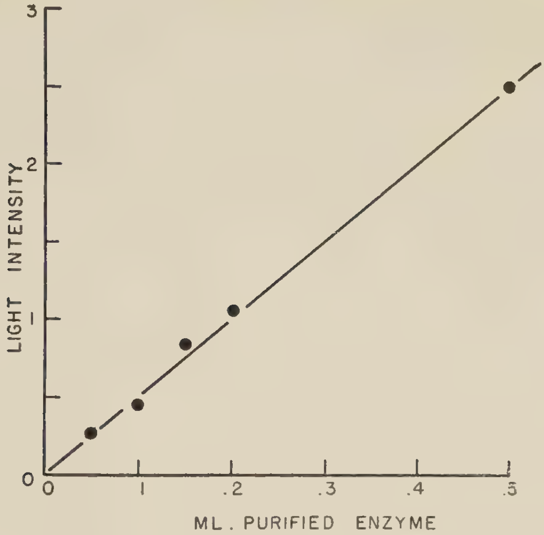

Fig. 7 Relationship of purified enzyme concentration to light intensity. Reaction mixture contained 0.6 ml 6.1 M NaCl, 0.5 ml heated crude, 0.2 ml 0.1 M tris-maleate buffer pH 6.6, and water and enzyme to a volume of 2.0 ml.

enzyme concentration upon initial light intensity with boiled crude extract (non-saturating concentration) added, indicating that no other factor was carried along in the enzyme fraction. The reaction with the purified enzyme appears to have the same characteristics as the crude extract insofar as the salt requirement, pH optimum and temperature coefficient are concerned.

Figure 8 shows the effect of different added amounts of a boiled crude extract upon the initial light intensity. The boiled crude extract appears to be relatively stable, but different preparations have given different values for maximum light intensity at saturation. This again suggests that inhibitors in crude extracts may influence the reaction, and that in such experiments they may have been present in greater or lesser concentrations.

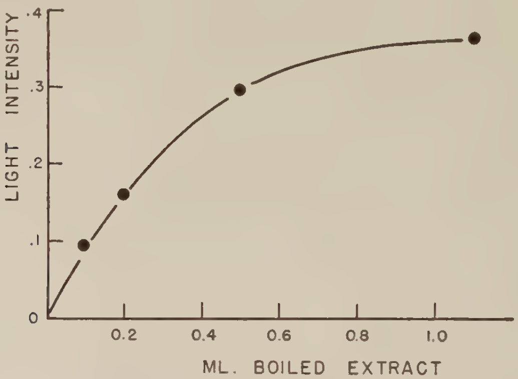

Fig. 8 Effect of the concentration of boiled crude extract upon luminescence of purified enzyme. Reaction mixture was composed of 0.6 ml 6.1 M NaCl, 0.2 ml 0.1 M tris-maleate buffer pH 6.6, 0.1 ml purified enzyme, and boiled crude extract and water to a volume of 2.0 ml.

Since it was thought that the decrease in activity of the crude extract (fig. 5) was not due to enzyme destruction, it was of interest to check this by determining the activity of an ammonium sulfate fraction from freshly prepared crude extract together with the activity of a similar fraction prepared from crude extract in which the activity had decayed

The activities before fractionation were in the ratio of 7.0 to 1.3 for fresh extracts and decayed extracts respectively, while the purified fractions had relative activities of 3.5 and 2.0 respectively, indicating that a major portion of the enzyme is recoverable after the decay of the crude extract.

The nature of the factor in the heat stable fraction has not been determined. In preliminary dialysis experiments it has not been possible to demonstrate any activity in the dialysate. A variety of cofactors, intermediates and other compounds have been tested with regard to their effect upon the reaction, and for their ability to replace this factor. None have been found to be active. Those tested include adenosine triphosphate, oxidized and reduced diphosphopyridine nucleotide, triphosphopyridine nucleotide, dodecyl aldehyde, oxidized and reduced flavins (riboflavin, riboflavin-5-phosphate, and flavin mononucleotide), thiamine pyrophosphate, coenzyme A, vitamin B_{12}, choline, acetylcholine, lipoic acid, liver and yeast concentrates, glucose, and glucose-6-phosphate. Crude homogenates of various organs of the rat and of the Pacific mussel were also tested and found to be either inactive or inhibitory. Irradiation with ultraviolet light, which has been shown to be effective in increasing enzyme activity in extracts of luminous bacteria, was found to have no similar effect upon the *Gonyaulax* enzyme. Cross tests between *Cypridina* luciferin and luciferase and similar *Gonyaulax* preparations did not give luminescence.

As mentioned previously, luminescence of the living organism is from 40 to 60 times greater at night than by day. Similarly, crude extracts prepared at night were found to be more active than those prepared in the daylight, but only by a factor of from 5 to 15 times. By ammonium sulfate fractionation it has been found that at least part of this difference in activity can be ascribed to enzyme concentration, and part to the activity of the heat stable fraction. In one experiment the crude extract from the night harvest was 8 times as active as the one from the daytime harvest. The purified enzymes from these fractions differed in activity roughly by a factor of 4 and the activities of the boiled crude extracts differed by a factor of about two, the fraction from the night harvest being more active in each case. Presumably therefore, both enzyme and substrate vary in concentration during the course of the day.

The decrease in luminescence with time in the reaction mixture is approximately logarithmic, suggesting that the reaction is first order in nature. In crude extracts the decay during the first two or three minutes is more rapid than during the later logarithmic period. In the purified extracts this phenomenon is much less marked. When $(NH_4)_2SO_4$ is used in place of NaCl in the reaction, the velocity constant is decreased by a factor of approximately 3/5.

It is of interest to mention that the classical luciferin-luciferase test does not give a positive reaction with *Gonyaulax* extracts. The test involves restoring luminescence to a reaction mixture which is dark following luminescence by adding an extract which was boiled before the decay of luminescence (see Harvey, '52). This is interpreted as a demonstration of the oxidation or utilization of a heat stable substrate, usually called luciferin, by the enzyme luciferase. The dark mixture contains only luciferase (the luciferin having been utilized in the luminescent reaction), and the boiled extract unoxidized luciferin. In the case of *Gonyaulax,* when boiled crude extract is added to a reaction mixture which is dim following luminescence a very slight (up to 10%) stimulation sometimes occurs, and at other times none at all. From the experiments with the purified enzyme it is clear that both an enzyme and a heat stable factor are involved in the reaction in a classical type luciferin-luciferase mechanism. It is not clear why the usual luciferin-luciferase test is negative, but the possibility that a product of the luminescent reaction itself is highly inhibitory is an attractive one. Extracts which have decayed spontaneously or those which have decayed with luminescence are inhibitory to the luminescent reaction, and the inhibition may be such that little or no response is obtained in the luciferin-luciferase test. It is also of interest that the living cell, which emits a flash upon stimulation, is rapidly (within 10–20 sec.) and almost completely fatigued with continuous stimulation. It is possible that this decrease in luminescence is due not to the exhaustion of the substrate but to the intracellular accumulation of the inhibitory products of the reaction.

DISCUSSION

A rhythmic expression of a variety of activities has been observed in a large number of organisms (Welsh, '38; Kleitman, '49). The present studies however, have provided evidence for the nature of the biochemical changes which may be correlated with the physiological rhythmicity of the organism. Although the variations in enzyme and substrate concentrations found in *Gonyaulax* occurred in phase with the diurnal changes in light intensity, there is good evidence from studies with living cells (Sweeney and Hastings, '57) that similar changes occur when the organisms are kept in the dark. Cells kept in continuous darkness show persistent fluctuations in light intensity. In preliminary studies it has been found that these fluctuations are correlated with similar changes in the activities of the extracts. The rhythmicity is therefore evidently at least partially the result of changes in concentration of the luminescent system. Further studies should provide information relevant to the general problem of the mechanisms controlling enzyme synthesis and destruction.

Certain properties of *Gonyaulax* extracts suggest that the reaction is a relatively simple oxidation such as occurs in *Cypridina,* where the reaction is often written as

$$\begin{aligned} LH_2 + \tfrac{1}{2}O_2 &\xrightarrow{\text{luciferase}} L^* + H_2O \\ L^* &\xrightarrow{\hspace{4em}} L + \text{light} \end{aligned} \qquad (1)$$

where LH_2 is the reduced substrate, luciferin, present as such in the extracts. When luciferin is oxidized in the presence of the enzyme (luciferase) an excited molecule results (L^*) which subsequently falls to the ground state with the emission of a quantum of light. All three bioluminescent reactions which have been studied are known to involve such a terminal oxidative reaction, which is characteristically very rapid (Hastings, McElroy and Coulombre, '53; Chance, Harvey, Johnson and Millikan, '40; Strehler and Cormier, '54; Hastings, '55). In both the firefly and bacterial extracts however, there are dark reactions preliminary to the oxidative step which are relatively slow, resulting in high overall

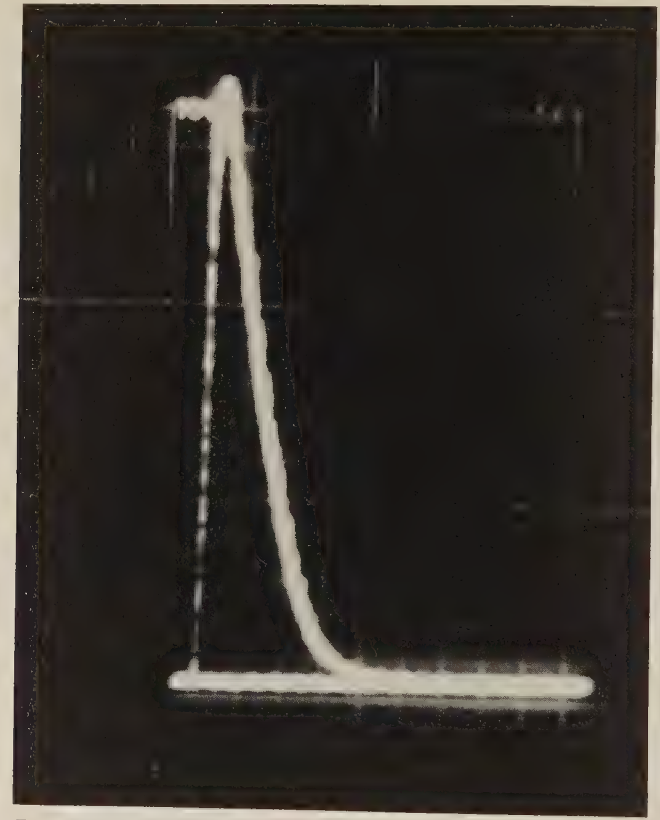

Fig. 9 Luminescent flash of *Gonyaulax* in response to mechanical stimulation, as photographed from the oscilloscope screen. Ordinate light intensity, abscissa time. Sweep time, 0.25 sec. One small division equals 0.025 sec. A test tube containing 5 ml of culture (about 1000 cells/ml.) was placed in front of the phototube and tapped very lightly. The flash is presumably the response of a single cell or of several cells flashing simultaneously.

activation energies (Strehler, '55; McElroy and Strehler, '49). Since the activation energy of *Gonyaulax* luminescence is lower (somewhat higher than *Cypridina),* it suggests that only a terminal rapid oxidation is involved in the luminescence of the extracts. If, as is possible, additional reactions preceding the oxidation are involved, they must be very rapid. The logarithmic decay is also in agreement with such a mechanism.

The blue color of the light is very similar to that of *Cypridina.* Since no light was obtained when *Cypridina* luciferin was added to crude or purified *Gonyaulax* enzyme, it must be concluded that the luciferins are different, even though it is possible that the mechanisms may be similar. It also seems certain that neither bacterial nor firefly components are involved in the reaction. It seems that not only has luminescence evolved independently in a great variety of unrelated organisms, but also, as Harvey ('53) has pointed out, that a considerable number of different luminescent systems have arisen independently.

The very rapid flash of *Gonyaulax* (fig. 9) is an excellent example of a precisely controlled biological reaction, and it is most logical to consider it as a type of cellular excitation. The mechanism by which an enzymatic reaction may be so regulated constitutes a major problem in modern physiology and biochemistry. As pointed out by Harvey ('52), the unicellular luminescent forms may be of great value in the study of these fundamental cellular processes.

SUMMARY

The extraction, partial purification and properties of the luminescent system in the marine dinoflagellate *Gonyaulax polyedra* are described. Crude cell-free water extracts of acetone powders emit light in the presence of salts, such as NaCl. The reaction also requires oxygen, and shows an optimum activity at pH 6.6 and 24°C. The light is blue-green with the emission peak at 478 mμ. The enzyme, purified by ammonium sulfate fractionation, requires for luminescence a

factor present in boiled crude extracts. It is suggested that this factor may be designated as *Gonyaulax* luciferin. The reaction appears to involve an oxidation of the luciferin. The intensity of luminescence of living cells shows a type of diurnal rhythmicity. The amount of both luciferin and luciferase which can be extracted from cells is correlated with these rhythmic intensity fluctuations. This phenomenon, as well as the mechanism of the reaction, is discussed.

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THE ACCUMULATION OF TISSUE COMPONENTS IN THE LEG MUSCLE OF THE DEVELOPING CHICK[1]

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TWO FIGURES

In the present study[2] it was attempted to define, for a limited period of development, the changes in the total mass of a specific tissue in terms of the quantitative changes of its components. Specifically, it was attempted to define the increase in the fresh weight of the leg musculature of the developing chick by the changes in the magnitude of the "extracellular" space, the cell number, and the dry weight of this tissue. These data are used for comparison with the rates of accumulation of certain cellular proteins of the developing muscle cell which were reported previously.

MATERIALS AND METHODS

Fertilized eggs were obtained from two commercial hatcheries. In the beginning eggs from Rhode Island Red chickens were more readily available and were therefore used as experimental material. Later on hybrid chickens, a cross of Delaware × New Hampshire strains, became the main source of

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[2] This paper is an extension and fuller documentation of a preliminary report presented at the Third Medical Conference of the Muscular Dystrophy Association (Herrmann, '55).

our fertilized eggs. The incubation was carried out in our laboratory but the record of the eggs preliminary to incubation (time, temperature) could not be exactly established.

Determination of fresh weight and dry weight. The entire leg was severed from the body in the region of the sacroiliac joint and the leg musculature of the embryos was carefully dissected from the tendinous insertion on the foot up to the insertion on the pelvic skeleton. The dissected musculature was promptly weighed, and then dried to constant weight at 96°C.

Number of cell nuclei. As an index of the number of cell nuclei the deoxyribosenucleic acid (DNA) content of the developing muscle tissue was determined using the procedure of Schneider ('45). For the determination of the amount of DNA per nucleus the following procedure was used: 50 mg portions of muscle were homogenized for one minute in a Virtis Micro homogenizer in 1.5 ml of an isotonic citrate sucrose buffer of pH 2.8. Six homogenate portions were combined and the nuclei were separated from the unbroken cells and the smaller particles by centrifugation. The nuclei were resuspended in buffer solution and aliquots were used for determination of the number of nuclei in a blood counting chamber and for determination of DNA respectively. Eight separate counts were carried out for each sample and the DNA was determined on 4 separate aliquots. A total of 32 samples were worked up in this way using muscle from chick embryos from the 11th to the 17th day of development. In spite of the precautions taken the obtained values for the DNA content per nucleus ranged from 2.20 to 3.96 μ with a mean of 2.98 $\times 10^{-6} \pm 0.07$ μ which is only slightly higher than the value given by Davidson and Leslie ('50).

Determination of inulin and chloride space. The distribution of inulin between the blood plasma and the muscle of chick embryos and freshly hatched chicks was determined after injection of known amounts of inulin into the circulation. The quantities of injected inulin were 0.1 ml of a 10% solution for the 10th–12th day; 0.1 ml of a 15% solution for the 13th day; 0.1 ml of a 20% solution for hatched chicks. The inulin was dissolved by immersion of the suspension into a boiling waterbath for three minutes. The injection procedure for embryos was introduced at this laboratory by Dr. I. R. Konigsberg and will be described in more detail elsewhere. A similar technique was used by Goldwasser and Shelesnyak ('53). In the hatched chick the inulin solution was injected into the wing vein. Before the embryos were removed from the egg, blood samples of about 0.2 ml were removed from the injected vein with the bent, sharpened tip of a thin glass pipette. The blood was pipetted into 2 ml centrifuge tubes in which 0.1 ml of a 2% oxalate solution had been dried. In the hatched chick the blood was collected from the decapitated animal. Aliquots of 0.1 ml of plasma and about 200 mg of muscle were placed into 18 mm × 150 mm pyrex tubes for the actual inulin determination. With each series of injected animals, muscle and plasma from uninjected animals to which known amounts of inulin were added was carried along. In addition, 4 standard solutions of inulin were carried through the entire procedure. The inulin determination was carried out following essentially the procedure described by Ross and Mokotoff ('51). In our experiments the volumes of reagent solutions were scaled down by a factor of 4 since the amounts of muscle remained near 200 mg. Neither plasma nor muscle blanks were found to give significant "inulin" values but inulin standards added to uninjected muscle gave final readings about 15% higher than the control standards. Therefore, the inulin content in muscle from inulin injected animals were always calculated from standards to which an equal amount of muscle from uninjected animals of the same age was added. This precaution was not necessary in the case of plasma the presence of which did not influence the readings of inulin standards. It was found necessary to clean the glassware by submerging in conc. tech. HCl over night. A maximum inulin level in the muscle was reached 60 minutes after injection and a plateau was maintained for at least two hours. Most of our samples were taken between 1½–2 hours after injection.

The determinations of chloride in muscle and plasma was carried out following closely the method described by Keys ('37).

Histological methods. A series of determinations of the extracellular space were carried out on sections of pieces of muscle tissue of 1–2 mm overall dimensions which were fixed in osmic acid solutions of 0.5%, 0.1% and 2.0% concentration, buffered to give a pH from 7.0–7.3. The fixation time was varied from 15 minutes to one hour without marked effect. After dehydration with graded concentrations of ethanol the tissue was embedded in methacrylate and cut at 0.5 μ, 1 μ and 2 μ thickness and stained with Wright's hematoxylin-eosin. This staining procedure was not adequate for detailed histological study but gave sufficient contrast to carry out the counts of histological elements on the sections following the method of Chalkley ('43–'44) as used by Peters and Flexner ('50).

The counts of cells in mitosis were carried out in 5 μ sections obtained after fixation in Bouin's solution and stained with hematoxylin-eosin. The differential counts of myoblasts and fibroblasts were carried out in 5 μ sections after staining according to the Van Gieson technique.

RESULTS

Fresh weight. The increase in fresh weight is used most frequently for the measurement of "growth" in biology. The fresh weights for the leg musculature of two strains of chick from the 8th to the 63rd day of development are given in table 1. The one set of determinations (I) was carried out during a single summer period using chicks of the Rhode Island Red strain while the measurements comprising the second series (II) were carried out at different seasons and during a time span of about two years using chicks of a New Hampshire × Delaware cross. During a part of the observed developmental period (12th–18th day) somewhat higher values were found in series I. This disparity is not necessarily an

TABLE 1

Fresh weight of leg musculature of the chick for a period of development from the 8th day to the 63rd day after start of incubation

SERIES I				SERIES II							
Age	No. of animals	Fresh weight	S. E.	Age	No. of animals	Fresh weight	S. E.	Age	No. of animals	Fresh weight	S. E.
days		*mg*		*days*		*mg*		*days*		*mg*	
8	10	19	± 1.1					24	15	1600	± 92
9	12	38	± 1.2					25	6	1510	± 56
10	12	67	± 5.4	10	26	54	± 2.5	26	13	1500	± 63
11	11	152	± 8.4	11	43	102	± 3.2	28	8	1510	± 115
12	4	181	± 5.0	12	57	131	± 5.6	29	38	1800	± 45
13	14	290	± 15.0	13	44	244	± 7.5	31	10	3440	± 310
14	12	429	± 13.2	14	44	272	± 11.5	32	7	3375	± 140
15	12	539	± 23.2	15	55	386	± 10.4	37	19	6155	± 150
16	10	602	± 19.3	16	37	478	± 10.0	40	6	8060	± 670
17	16	745	± 29.1	17	18	668	± 29.2	42	11	10910	± 380
18	12	968		18	25	889	± 35.6	45	6	12435	± 680
19	12	1007		19	21	1010	± 31.7	51	12	21550	± 1300
20				20	6	1150	± 30.5	53	6	25650	± 1400
21				21	4	1320	± 41.5	59	4	29100	± 1200
				22	6	1340	± 75.2	63	6	47400	± 2800
				23	12	1700	± 35.4				

inherent characteristic of the two different strains since the development of fertile eggs obtained from commercial sources may be influenced by uncontrolled environmental factors.

It can be seen that the weight accumulation is not continuous throughout since a slight drop in the fresh weight occurs shortly after hatching, caused probably by inadequate food and water uptake of the freshly hatched chick. Apart from this relatively small irregularity the overall course of the curve shows an increasing steepness of its slope.

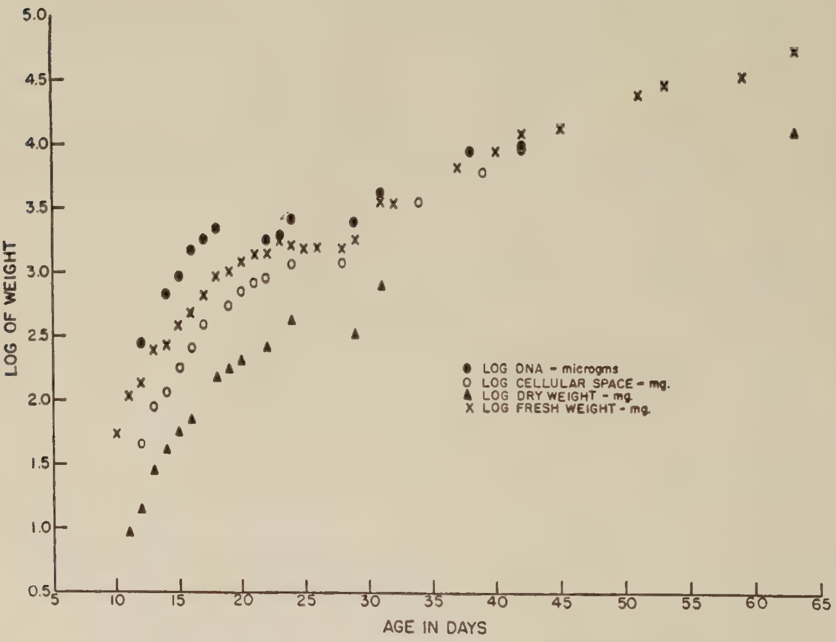

Fig. 1 Logs of fresh weight, dry weight, cellular space and deoxyribosenucleic acid found in the total leg musculature of the chick for the development from the 10th to 63rd day.

The log plot of the fresh weights shows that the increase in absolute growth increments is not an exponential one since the log plot of the fresh weights gives a curve with a decreasing slope (fig. 1) indicating a gradual decrease in the relative growth rate. An increase of the absolute weight gains simultaneous with a decrease in the relative growth rate can occur only in that part of a sigmoid growth curve which follows the initial, nearly exponential, portion and precedes the linear part in the region of the inflection point of this curve. This shows that the observed period of accumulation of muscle fresh weight comprises only a small sector of a complete sigmoid growth curve. The same is indicated by a consideration of the growth curve for the total body weight of chickens. According to the data compiled by Brody ('45) the total body weight of fowl reaches the halfway point of growth on the 6th month. Since muscle represents an important portion of the total body weight it can be assumed that muscle growth follows a similar course and that therefore the period of about 55 days, investigated in this study, is only about ⅙th of the total growth period. For this reason and in view of the fact that this period represents a rather ill-defined transitional phase of the sigmoid curve the application of one of the mathematical representations for whole growth curves seemed problematical. Instead it was attempted to interpret the accumulation of the muscle fresh weight in terms of the accumulation of some of its components.

Dry weight. Similar to the accumulation of fresh weight the increase in dry weight shows a slight difference in the values obtained in the two series of measurements (table 2). The log plot carried out only for series II indicates again a gradually decreasing slope during the development from the 8th to the 24th day (fig. 1). The total dry weight accumulation is about twice as fast as the accumulation of fresh weight as indicated by an increase in dry weight from 8% to 17% of fresh weight during development from the 11th to the 20th day. After hatching the ratio between fresh weight and dry weight remains essentially constant (22%) as borne out by comparison of the values for the 22nd, 24th, 29th, 31st and 63rd day of development.

Extracellular space. In the mature animal the extracellular space takes up a relatively small and constant portion of the total tissue volume. In the muscle of the young chick the extracellular space has been shown to be higher than in the mature animal (Barlow, Slinger and Manery, '47; Barlow and Manery, '54), and a similar observation has been made (Flexner and Flexner, '49) for the central nervous system of the guinea pig embryo. These findings made it apparent that the magnitude of the extracellular space had to be determined as one of the variable components of the total fresh weight of a developing tissue.

TABLE 2

Accumulation of dry weight in developing chick muscle

	SERIES I			SERIES II		
Age	No. of animals	Total dry weight	Dry wt./100 mg fresh weight [1]	No. of animals	Total dry weight	Dry wt./100 mg fresh weight [1]
days		*mg*	*mg*		*mg*	*mg*
8	10	2.3 ± 0.15	12.1			
9	12	3.9 ± 0.12	10.3			
10	12	6.8 ± 0.39	10.1			
11	11	13.4 ± 0.63	8.8	12	8.9 ± 0.6	8.7
12	4	15.9 ± 0.27	8.3	16	14.6 ± 1.3	8.2
13	14	29.0 ± 1.8	10.0	4	29.3 ± 3.3	10.0
14	12	59.0 ± 2.6	13.6	12	42.8 ± 1.6	11.3
15	12	70.3 ± 3.4	13.0	20	60.3 ± 2.3	13.4
16	10	88.6 ± 4.0	14.6	19	71.6 ± 2.9	14.5
17	16	117.1 ± 3.8	15.7			
18	12	166.5 ± 4.2	17.2	17	151.5 ± 5.7	16.3
19	12	178.7 ± 5.7	17.7	8	172.2 ± 14.4	17.4
20				6	202.3 ± 7.7	17.6
22				6	264.1 ± 12.7	19.7
24				6	426.6 ± 24.6	22.2
29				5	336.3 ± 41.2	19.7
31				10	779.4 ± 86.5	23.0
63				6	10,900.6 ± 57.8	22.9

[1] Means of dry weight in per cent of fresh weights calculated for individual samples.

The use of chemical methods for the determination of the extracellular space (ES) is based on the assumption that the permeability properties of cells prevent certain substances, e.g. chloride or inulin from entering the cells and that they

TABLE 3

Chloride in plasma, breast muscle and leg muscle of chicks at different stages of development

	PLASMA		BREAST MUSCLE			LEG MUSCLE			
								Extracellular space	
Age	No. of animals	Chloride content	No. of animals	Chloride content	Extracellular space in % of fresh weight	No. of animals	Chloride content	In % of fresh weight	In total leg muscle
days		*m.eq.Cl/1000*		*m.eq.Cl/kg*			*m.eq.Cl/kg*		*mg*
10	8	122.0 ± 5.8	..	..	...	6	110.5 ± 5.1	86	46.4
11	6	126.1 ± 10.4	..	..	...	14	88.7 ± 4.3	67	68.3
12	6	112.9 ± 2.0	3	76.8 ± 1.3	68.6	6	78.4 ± 1.8	66	86.5
13	9	113.3 ± 3.5	2	81.9 ± 0.6	68.7	15	81.0 ± 4.5	68	165.9
14	6	111.1 ± 2.9	6	77.1 ± 3.7	66.0	6	78.1 ± 1.9	67	182.2
15	7	106.6 ± 5.5	4	72.3 ± 3.3	64.4	8	65.4 ± 5.0	58	223.9
16	4	104.9 ± 2.6	4	60.7 ± 3.0	55.0	4	52.5 ± 2.2	48	229.4
17	6	120.0 ± 8.7	7	54.3 ± 4.4	50.6	7	46.0 ± 2.6	43	287.2
18	..	..	3	61.0 ± 2.4	52.2	..	..	..	....
19	4	111.0 ± 3.0	..	..	...	3	61.0 ± 2.5	47	474.7
20	2	98.5 ± 0.6	2	50.8 ± 0.55	49.0	2	40.0 ± 0.4	39	448.5
21	13	117.9 ± 7.4	..	..	...	14	47.6 ± 8.1	38	501.6
22	6	112.3 ± 2.5	8	69.4 ± 8.4	55.9	6	38.6 ± 2.2	33	442.2
24	3	111.7 ± 1.0	3	49.5 ± 3.3	42.1	3	30.9 ± 2.9	26	416.0
34	3	121.2 ± 2.6	2	25.5 ± 2.3	20.0	3	31.6 ± 1.9	25	1040.0[1]
39	4	122.0 ± 2.5	3	23.8 ± 4.6	18.5	4	28.6 ± 5.6	22	1635.0[1]
42	4	125.0 ± 1.4	4	20.9 ± 0.4	15.9	4	27.7 ± 1.7	21	2291.1

[1] The fresh weight values were interpolated graphically.

remain confined to the extracellular compartment of the tissues in a concentration equal to that found in the blood plasma. Since little is known about the permeability of embryonic cells it seemed advisable to use for measurements of the ES in embryonic tissues two different substances and to check the results obtained in this way by an independent histological procedure. For the chemical determination of the extracellular space in muscle of developing chick analyses were carried out of the distribution of chloride (table 3), and of inulin (table 4) in the blood plasma and in the muscle of developing chick, and in addition, it was attempted to assay the ES in histological sections. From the values for ES the magnitude of the cellular space (CS) could be calculated (fig. 2).

TABLE 4

Inulin in plasma and leg muscle after injection into chick at different stages of development

AGE	NO. OF ANIMALS	AMOUNT OF INULIN INJECTED	INULIN IN PLASMA	INULIN IN LEG MUSCLE	EXTRACELLULAR SPACE	
					In % of fresh weight	In total leg muscle
days		*mg*	*µg/0.1 ml*	*µg/100 mg*		*mg*
10	3	10	22.0 ± 0.5	18.1 ± 0.5	82	44.3
12	10	10	17.5 ± 1.2	11.2 ± 0.6	64	83.8
13	17	15	20.5 ± 0.5	11.9 ± 0.4	58	141.5
14	7	20	25.2 ± 1.5	12.6 ± 0.9	50	136.0
15	6	20	21.8 ± 1.5	10.7 ± 0.4	49	189.1
17	9	20	17.5 ± 0.8	7.3 ± 0.3	42	280.1
28	6	100	9.0 ± 1.8	2.0 ± 0.3	22	332.2

The values which were obtained for the inulin and chloride contents show that the changes in the distribution of the two substances between muscle and plasma follow a similar pattern. Only from the 13th to the 17th day the ES as derived from inulin distribution seems to decrease somewhat faster than is indicated by chloride determinations. If it is assumed that the embryonic cell is impermeable to chloride, or inulin, values for ES would be obtained which are somewhat higher than 80% on the 10th day, drop to about 60% on the 12th day and gradually decrease after this day to a value of about

25% on the 25th to 28th day of development. It should be mentioned that the decrease of the ES in different muscles does not follow an identical course. This is apparent from the comparison in table 3 of the chloride values for ES in leg muscle and in breast muscle. The values for breast muscle are in good agreement with the data given by Barlow, Slinger and Manery ('47) and Barlow and Manery ('54) who attempted to substantiate the assumption concerning the chloride distribution of tissues by equilibration experiments.

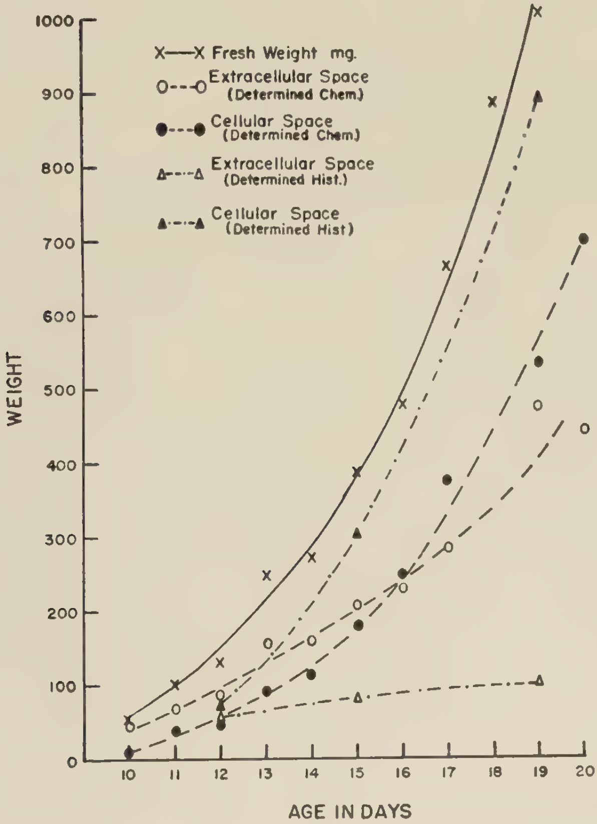

Fig. 2 Values (in milligrams) for extracellular space determined chemically and histologically and derived values for the cellular space.

The observation of an apparently greatly expanded ES in the earlier phases of development would not seem surprising since the muscle tissue of the 12th to 14th day-old embryo is of a gel-like consistency with a high content of water and only 8%–10% dry matter. However, from determinations of the dry weight it becomes evident that an ES of 80% corresponding to a cellular space (CS) of 20% would imply a concentration of dry matter in the cellular space amounting to 60%. This is a value which is incompatible with all known data about dry matter content of tissues. Therefore, one has to conclude that at this early stage of development the cells of muscle tissue must be permeable to both chloride and inulin giving rise in this way to apparently excessive values for the ES and, correspondingly, too low values for CS. On the 12th day the concentration of dry matter in the CS has dropped to 30% remaining at a level of 25% up to the time of hatching. A dry weight content of CS on the 12th day apparently equal to that of the hatched chick does not necessarily mean that by that time the permeability properties are approaching the mature state. A constancy of the ratio CS/dry matter could be maintained by a simultaneous increase in the concentration of dry matter within CS and an apparent simultaneous increase in the CS due to a diminishing permeability of the cells for inulin and chloride.

The values for the chemically determined extracellular space (ECS) differ greatly from the values obtained by a quantitative assay of histological sections (ESH). Because of the laboriousness of the histological method determinations were carried out for the muscle tissue only from single embryos at the 12th, 15th, and 19th day of development giving an ESH of 42% (1311 counts), 21% (1896 counts), and 11% (3534 counts) respectively. At all three ages the results of the histological determinations were considerably lower than the figures obtained by chemical methods. It should be pointed out that the relative difference in the results obtained with the two methods is at least as great on the 19th day of development as on the 12th day in spite of the fact that the content of dry matter about doubles during this time span reaching almost adult level. This makes it unlikely that excessive shrinkage due to low content of dry matter is the cause of this discrepancy, particularly, since an attempt was made to minimize shrinkage in the tissue by using osmic acid for fixation and methacrylate as imbedding medium. In contrast to our results no discrepancy between chemically and histologically determined space has been found in the muscle of hatched chick (Barlow and Manery, '54). To what extent this difference can be attributed to the use of different histological techniques has not been investigated.

The results of both methods show not only qualitatively that the ES decreases and CS increases considerably during the investigated part of muscle development. Our data also show that quantitatively the relative changes of the CS values observed with the two methods are quite comparable. For example, the ratios of the CS found between the values for the 16th and 12th days are 5.3, 5.7, and 5.2 for the inulin space, chloride space, and histological space respectively, and a similar agreement is found for increases in the chloride space and histological space during development from the 16th to the 20th day (2.8 × and 2.6 × respectively). This shows that in spite of the wide disparity of the absolute values for ES obtained by the two methods, the relative increase of the cellular spaces provides a consistent parameter. The comparison of the increase of other magnitudes is carried out in a later section of this paper.

Cell number. In most embryonic tissues a part of the increase in mass is due to cell division. However, in muscle in which the fibrillar structure is maintained throughout a large part of development, a rapid nuclear multiplication can be frequently observed without a corresponding typical division of the cytoplasm. In this case, it is merely the quantity of cytoplasm per cell nucleus which is comparable to the histologically discernible protoplasmic units of the cell and the increase in the number of cell nuclei is considered to be equivalent to the production of new nucleo-cytoplasmic units.

The increase in the number of cell nuclei was measured by the determination of deoxyribosenucleic acid (DNA). This procedure, which has been widely accepted, presupposes that the amount of DNA per cell nucleus remains constant during the investigated period of development. That this seems to be the case is indicated by the work of Davidson who finds about 2.6×10^{-6} micrograms DNA per cell nucleus of the adult or embryonic muscle cell of the chick (Davidson and Leslie, '50). However, in a recent paper by Nowinski and Yuschok ('53) a value of about 8×10^{-6} micrograms is given for cell nuclei of the chick leg. A reexamination of the DNA content per nucleus of chick muscle showed no significant deviation from an average value of 2.98×10^{-6} micrograms DNA/nucleus during the 11th–17th day period of development. In attempting to explain the values found by Nowinski and Yuschok it should be pointed out that these investigators homogenized with a Potter homogenizer which leads to the destruction of a large portion of nuclei and to the accumulation of much nuclear debris. Unless this nuclear debris is

TABLE 5

Desoxyribosenucleic acid (DNA) content of the leg muscle of developing chick

AGE	NO. OF ANIMALS	DNA PER 100 MG FRESH WEIGHT	DNA IN TOTAL LEG MUSCLE
days		*µg*	*µg*
12	16	212 ± 6.6	278
14	8	242 ± 2.0	656
15	6	252 ± 1.7	942
16	6	306 ± 4.9	1460
17	8	275 ± 3.2	1840
18	8	240 ± 1.8	2140
22	6	130 ± 1.8	1750
23	6	108 ± 1.6	1830
24	9	151 ± 3.1	2510
29	6	129 ± 0.5	2320
31	8	121 ± 9.5	4000
38	4	118 ± 4.8	8200
42	5	85 ± 2.7	9300

carefully removed it increases the DNA values without affecting the counts giving rise to excessive values for the quantity of DNA/nucleus.

From the data listed in table 5 one can see that the accumulation of DNA from the 12th to the 16th day follows closely an exponential course since the log plot (fig. 1) for this period is practically linear. After the 18th day no further accumulation of DNA occurs for a period of about 10 days except for a somewhat erratic peak around the 25th day of development. After the 29th day the DNA shows a marked increase which gradually diminishes after the 35th day.

If calculated in per cent of fresh weight the DNA content of muscle was found to increase to a maximum from 212 mg % on the 12th day to 306 mg % on the 16th day. The occurrence of a maximum for the DNA/fresh weight on the 16th day was observed in one set of previous analyses (Konigsberg and Herrmann, '55), but it was found to be absent in another series of determinations (Herrmann, '52). In the latter series, breast muscle of the chick embryo was examined and it has to be concluded that in this muscle the DNA/fresh weight ratio remains more constant than in the leg muscle of the chick embryo. Possible reasons for this difference are discussed below.

MITOTIC ACTIVITY

Parallel with the determination of total DNA the incidence of mitosis was investigated as a possible index for the increase in the total number of cell nuclei. According to the figures listed in table 6 the number of cells in mitosis (in per cent of total cell number) declines from 1.5% on the 8th day to about 0.06% on the 21st day. After that time the rate remains about the same until the 24th day (0.07%). It should be noted that mitotic activities were about the same irrespective of whether the chicks were killed during the day or during the night. This makes it unlikely that the observed decrease of mitotic cells is due to a shift in the diurnal rhythm of mitotic activity if such exists at these stages of development (Jaffee, '54).

TABLE 6

Incidence of mitoses in leg muscle of developing chick

The counts are given for muscle from individual animals

AGE													
8 Days		11 Days		14 Days		16 Days		18 Days		21 Days		24 Days	
Total count	Per cent mitosis	Total count	Per cent mitosis	Total count	Per cent mitosis	Total count	Per cent mitosis	Total count	Per cent mitosis	Total count	Per cent mitosis	Total count	Per cent mitosis
6,500	1.4	11,900	0.79	9,600	0.50	9,800	0.49	10,100	0.29	9,800	0.07	8,600	0.05
4,900	1.7	8,700	0.93	14,200	0.41	12,500	0.32	10,300	0.14	12,100	0.07	7,400	0.09
3,200	1.5	9,000	0.64	14,400	0.24	11,300	0.23	10,700	0.24	12,000	0.03	8,700	0.14
6,300	1.4	14,500	0.85	11,700	0.48	9,300	0.37			10,200	0.03	7,600	0.07
4,900	1.5	15,300	0.80	8,500	0.58	13,600	0.39			11,500	0.07	7,100	0.08
				15,500	0.45	10,700	0.36			12,100	0.008	5,400	0.07
										7,900	0.06	4,900	0.06
										10,200	0.00[1]	9,300	0.06
										6,100	0.05[1]		
										6,100	0.05[1]		
										7,100	0.01[1]		

[1] Samples obtained about midnight; the other samples were obtained during day-time.

From the incidence of mitosis the daily increase in the number of cell nuclei was approximated. For this purpose a definite time (t in hours) for the duration of a mitotic cycle had to be postulated. If it is observed that a certain percentage (p) of all cells are in mitosis during this time (t) the per cent of cells which will duplicate during a day will be (p × 24/t). Since the most direct observations of dividing chick fibroblasts give a duration for the mitotic cycle of 30 minutes (Olivo and Slavich, '30), this figure was used as the basis for one set of calculations. Considering the possibility that nuclear proliferation in muscle cells may occur at a faster rate calculations for a 15 minute duration were also

TABLE 7

Calculation of number of cell nuclei and of DNA based on incidence of cells in mitosis given in table 6

AGE	NUMBER OF CELLS IN MITOSIS	DAILY PER CENT INCREASE OF CELL NUCLEI		CALCULATED DAILY INCREMENT IN DNA		CALCULATED AMOUNT OF DNA		DNA CONTENT FOUND BY DIRECT MEASUREMENT
		I	II	I	II	I	II	
days	%	µg		µg		µg		µg
8	1.5	72.0	144.0					
9	1.3 [1]	62.3	124.6					
10	1.1 [1]	52.8	105.6					
11	0.90 [1]	43.2	86.4					
12	0.80	38.4	76.8	107	214	278	278	278
13	0.62 [1]	29.8	59.6	115	293	385	492	
14	0.45	21.6	43.2	108	339	500	785	656
15	0.40	19.2	38.4	118	394	608	1024	
16	0.35 [1]	16.8	33.6	120	480	726	1418	1460
17	0.28 [1]	13.9	27.8	117	528	846	1898	
18	0.22	10.6	23.2	102	564	963	2426	2140
19	0.16 [1]	7.7	15.4	82	460	1065	2990	
20	0.11 [1]	5.3	10.6	61	365	1147	3450	
21	0.06	2.9	5.8	35	221	1208	3815	
22	0.07 [1]	3.4	6.8	42	274	1243	4036	1750
23	0.07 [1]	3.4	6.8	44	293	1285	4310	
24	0.08	3.8	7.6	51	350	1329	4603	2510

[1] Indicates values obtained by arithmetic interpolation.

Calculations in columns I are based on a duration of mitosis of 30 minutes.

Calculations in columns II are based on a duration of mitosis of 15 minutes.

carried out. The increases in cell number, found in this way, are listed in table 7, columns 3 and 4. The daily figures for the per cent increase in the number of cell nuclei were then used to calculate the derived increments in the DNA content after the 12th day for a starting value of 278 micrograms (columns 5 and 6). Addition of the daily increments give the total calculated amounts of DNA (columns 7 and 8) which can be compared with amounts of DNA found in the chemical analyses (column 9). From this comparison it can be seen that during development from the 12th to the 18th day the values for the DNA content derived from the mitotic rate are only about one-half the chemically determined values if calculated for a mitotic period of 30 minutes but are in close agreement if calculated for a mitotic period of 15 minutes. This agreement may be fortuitous in view of the fact that the shortest time for a mitotic cycle of cells of the chick embryo is 30 minutes and that shorter times have been observed only in two unrelated species (Hughes, '52). If it is assumed that a 30 minute mitotic cycle is closer to the actual duration of a mitotic cycle an alternative explanation of the discrepancy of the calculated and observed DNA values would be necessary. It should be mentioned that several authors have postulated nuclear divisions in the muscle cell which do not exhibit the typical picture of a mitotically dividing nucleus using occasionally the term ''amitotic'' division to designate this process (Hughes, '52; Le Gros Clark, '46). Such atypically dividing nuclei would not be included in the mitotic counts and would give too low values for the rate of nuclear proliferation. For the period following the 18th day the calculations for both mitotic periods (30′ and 15′) give values which are in disagreement with the actually found values for DNA. Mitotic activity even if calculated for the longer mitotic period would indicate an increase in newly formed cells of about 50% to 60% during this period and a correspondingly larger accumulation if the calculation is carried out for the shorter mitotic period. On the other hand, it was noted previously that the DNA when determined chemically was found to accumulate only by a total of 10% during the period from the 18th to the 29th day of development. Only a slowing down of the mitotic period to about 2–3 hours would give agreement between these figures. Since such a change is unlikely one could suggest that the increase due to mitosis is compensated for by an equal decrease of nuclei due to degeneration.

CELL NUMBER AND CELL SPECIES

The determination of the total cell number per fresh weight unit prompted the question of the proportion of cell nuclei contributed by the different types of cells which make up muscle tissue. In attempting to account for the two most prevalent types of cells e.g. fibroblasts and muscle cells differential counts were carried out on sections which were stained according to Van Gieson technique. The color difference was found to be pronounced in the chick at the 21st and at the 18th day of development but at the 16th day, or earlier, the difference was less distinct and a separation of the cells became unreliable. In carrying out the cell counts of sections obtained from three embryos on the 18th day and from 5 embryos on the 21st were examined. Out of a total cell count of 4718 for the 18 day embryos 611 cells were found to be fibroblasts (13%); and out of a total cell count of 3686 for the 21 day old embryos the number of fibroblasts were 533 (14.5%). Since there was no marked difference in the results obtained on these days we regard this figure as at least a measure of magnitude of the ratio of fibroblasts and myoblasts.

DISCUSSION

For the discussion of the quantitative relations of the measured parameters in muscle development the investigated growth period is divided into phases from the 12th–16th day, 16th–20th day, 20th–30th day, and 30th–50th day respectively.

12th–16th day period. During this period the fresh weight of the leg muscle of the chick increases from 131 mg to 478 mg that is by an accumulation factor of 3.7. At the same time the extracellular space was found to increase from 55 mg to 81 mg as measured histologically and from 85 mg to 220 mg when measured chemically corresponding to accumulation factors of 1.5 and 2.6 respectively. The cellular space increases from 76 mg to 392 mg (measured histologically) and from 47 mg to 354 mg (measured chemically) with accumulation factors of 5.2 and 5.3 respectively. To be compared with these data is the accumulation of DNA which is regarded to be equivalent to the increase in newly formed nucleo-cytoplasmic units in the muscle tissue corresponding to the proliferation of new cells in other tissues. From our compilation it is evident that the DNA accumulated from 278 micrograms to 1460 micrograms giving an accumulation factor of 5.3. It can be seen that the figures for the increase of cell space and cell number (DNA) are in close agreement indicating that the cell volume remains constant and that the increase in cell space is predominantly due to cell proliferation.

The dry weight increases during this period from 14.6 to 71.6 mg giving an accumulation factor of 4.9. This figure shows that the dry weight increases at about the same rate as the cellular space and the cell number and that therefore the concentration of dry matter in the muscle cell does not change significantly during this phase of development.

The data show furthermore that the components of the cellular compartments of the developing muscle (cell number, cell volume, dry matter) increase at a similar rate which is faster than the accumulation rate of fresh weight. The extracellular space, on the other hand, increases more slowly than the fresh weight. It is this divergence in the accumulation of the extracellular and cellular tissue compartments which explains the increase of dry weight and DNA content relative to the increase in fresh weight during this period.[3]

Compared with the tissue components discussed so far the rates of increase of the specific cell proteins, actomyosin and collagen, are of an entirely different order of magnitude during the period of development from the 12th to the 16th day. Using data reported previously (Csapo and Herrmann, '51; Herrmann and Barry, '55) a total accumulation from 194 to 2840 micrograms for actomyosin and from 91 micrograms to 2279 micrograms for collagen for the 12th to the 16th day period gives accumulation factors amounting to 15 to 25 respectively. In contrast, the activity of alkaline phosphatase, a more ubiquitous less specific protein, increases during this period from 655 activity units to 4400 activity units with an accumulation factor of 6.7. The specific proteins accumulate thus during this time at rates which are about 3 to 5 times greater than the accumulation of dry weight or of the bulk of proteins (unpublished data), and about 2–4 times faster than the accumulation of alkaline phosphatase.

Since the term differentiation indicates the appearance of specific cell products the rapid increase of actomyosin and collagen can be regarded as a quantitative index of differentiation during this period. It is of interest to point out that the rapid formation of specific proteins coincides with considerable proliferative activities. Since, in general, it is assumed that cell division and differentiation are mutually exclusive cell activities, our observation of simultaneous nuclear proliferation and differentiation would, therefore, seem paradoxical. However, it should be pointed out that we do not know as yet whether these two processes actually do occur in the same cell or whether only those cells form specific proteins in which proliferation has come to a standstill. Also, it has to be kept in mind that in the muscle nuclear proliferation is not accompanied by cytokinesis, and therefore, it seems possible that differentiation and proliferation could occur in the same cellular unit.

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[3] As mentioned in a previous section, analyses of breast muscle showed no increase in the ratio DNA/fresh weight. This must mean that in this tissue the "cell number" increased at the same rate as the fresh weight. If the cell size remains constant the increase in cell number, in total cell space, in total extracellular space, and in total fresh weight should be proportional. Since some decrease in ES was found it had to be assumed that an increase in cell size occurs in the breast muscle during this phase of development. An increase in cell size during this phase of development would be one of the properties which distinguish the breast muscle from the leg muscle of the chick embryo. An exploration of this assumption has not been successful, so far, because of the technical difficulties in determining the nucleocytoplasmic ratio in embryonic muscle tissue.

Period 16th–20th day. During this period the accumulation of the fresh weight and of all of its components is slower. The most marked decrease occurs in the accumulation of DNA in particular after the 18th day. In the preceding period DNA accumulation was close to CS accumulation but in this period the increase in DNA (cell number) is only one-half as fast as the accumulation of CS. This indicates that the ''cell volume'' must increase considerably (about 2 ×) which is evident from an inspection of histological sections. The increase in dry weight corresponds closely to the increase in CS indicating that the dry weight gain relative to fresh weight is due to the expansion of CS while the DW concentration in the CS remains about the same. The accumulation of specific proteins decreases likewise, more pronounced in the case of collagen.

Period 20th–30th day. During this period the accumulation of all components shows some irregularity due to the adjustments of the chick during the post-hatching period. The fresh weight does not increase from the 23rd to the 28th day. This is paralleled by cessation in the increase in the cellular space and the dry weight. The increase in DNA and phosphatase is already suppressed by the 19th day and remains on a very low level until the 28th day. The accumulation of actomyosin and of collagen, though temporarily decreased shows a much smaller deviation from the continuous course than fresh weight for example.

Period after the 30th day. During the final period a slow and uniform accumulation of the measured components occurs. In the absence of a differential increase in the cellular and extracellular space (according to Barlow, Slinger and Manery, '47) a slight excess in the accumulation of fresh weight over that of DNA shows that there is a continuation of the increase in "cell size." The rate of actomyosin accumulation, however, remains higher than that of the other components. In this sense the process of differentiation continues during this period.

From these observations it is apparent that the investigated period of development of the leg musculature is a truly transitional phase. It begins with the end of the purely proliferative increase of the cellular space and it ends with the beginning of the predominantly cytoplasmic expansion of the cellular space. Although this transitional phase is only a small portion, about ⅕th to $1/10$, of the entire span of the growth curve of the leg muscle the analysis of a few components of the tissue reveals several distinctive periods within this short time span during which changing and even divergent processes give rise to the overall picture of fresh weight accumulation ordinarily described as growth. Obviously, the data presented here give only a minimum number of parameters necessary for an analysis of the mechanism of muscle growth. This analysis is therefore being continued at this laboratory by a comparison of accumulation rates of proteins with their incorporation rates of labelled amino acids and by the analysis of the effects of physiological and non-physiological growth regulating substances on the accumulation of individual components of the growing system.

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SUMMARY

1. The total increase in the fresh weight of chick leg muscle during the development from the 12th–60th day has been expressed in terms of dry weight, extracellular space, cellular space and cell number and these growth parameters were compared with the increase in the specific proteins actomyosin and collagen.

2. It was found that the increase in fresh weight during the period from the 12th–16th day is due primarily to the increase in cell number. The cellular space, the cell number and the dry weight increase by about the same factor (4.9–5.3 times). The extracellular space increases only about one-half as fast (1.5–2.6 times). The increase in specific proteins, however, is considerably faster (15–25 times).

3. During the following period of development (16th–20th day) the increase of fresh weight diminishes primarily due to a decrease in cell proliferation. At the same time there is an increase in the amount of cytoplasm per nucleus.

4. During the last phase (after the 30th day) the increase in fresh weight is due almost entirely to an increase in the cytoplasm per nucleus with a concomitant increase in actomyosin.

5. The obtained measures give a quantitative description of the transition of the leg musculature of the chick embryo from a primarily proliferating into a differentiating state.

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THE INFLUENCE OF pH OF THE SUSPENDING BUFFER ON RESPIRATION OF APICAL SEGMENTS OF THE ONION ROOT

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A study of some of the literature with regard to the effect of hydrogen ion concentration on respiration, reveals that some investigators report maximum respiration at a given pH, while others observe either a stimulation or depression of respiration with either an increase or decrease in acidity. For example, Brooks ('21) measured the CO_2 production of two species of bacilli and found a maximum at a pH of 7.0 and 6.8 respectively. Steward and Preston ('41) working with potato discs, noted that respiration (as well as protein synthesis and bromide ion absorption) was at a maximum at a pH of 7.0 and decreased as the solution was made either more acid or alkaline. Lyon ('27) observed an increase in CO_2 output by *Elodea canadensis* as pH increased from 7.0 to 9.18, while, on the other hand, Thomas ('31) working with carrot tissue noted an increase in respiration with increasing acidity. Lemmon ('36) studying potato tissue found maximum respiration to result between pH's of 3.5 and 5.2 with a decrease in rate as the solution became either more alkaline or more acid. Also with potato tubers, Boswell and Whiting ('38) noted that oxygen uptake was essentially unaffected between pH's of 5.29 and 6.81. Oxygen consumption showed a slight decrease at pH values below 5.29 and an increase from pH 6.81 to 8.04.

Recently, Jeffrey ('51) has studied the effect of pH on the rate of oxygen uptake, and methylene blue reduction shown by onion roots. She found that oxygen consumption was not affected between pH values of 3.0 and 9.6. In solutions with pH's of 2.5 and 2.8 oxygen consumption was decreased. Jeffrey also noted that the rate of reduction of methylene blue by the onion root was not altered by prior immersion of the root for two hours in solutions with pH's between 4.0 and 9.6. However, if the roots were immersed in a solution of pH 2.0 they later failed to reduce methylene blue, and if immersed in a solution of pH 3.0 the apical 10 mm subsequently failed to reduce methylene blue.

In general, with regard to the effect on respiration, there appears to be a fairly large pH range over which change in pH does not alter gas exchange; exceeding this range usually results in a decrease in respiration. It should be noted however, that in most of the foregoing cases, only one component of respiration (i.e. either O_2 uptake or CO_2 output) was measured.

Bonner and Wildman ('46) have evaluated the influence of pH of the suspending buffer on the respiratory rate and respiratory quotient of spinach leaves. They found over a pH range of 4.5 to 7.0 that oxygen uptake was essentially unaffected, however at the higher pH values carbon dioxide output was decreased. For example, the R. Q. at pH 4.5 was found to be 0.93, while at a pH of 7.0, it was only 0.73. Bonner ('34) had previously noted the same phenomenon with regard to respiration of avena coleoptiles. These authors point out that as yet no explanation has been offered to account for this decrease in CO_2 output at higher pH values.

In view of these facts, it seems likely that the pH of the suspending buffer might exert some influence upon gas exchange of apical segments of the onion root. This merits investigation because in past studies of onion root respiration the Warburg manometric technique has been used extensively, and in most of the measurements the tissue was suspended in M/15 phosphate buffer at a pH of 4.5 (Berry, '49; Berry and Norris, '49; Norris, '51; et al.). This was obviously done as a matter of practicability, in that when measuring carbon dioxide production, combined CO_2 does not have to be liberated from the suspending liquid if the pH is maintained at 4.5. Also in some of the past measurements (Norris, '51), respiratory quotients have run fairly high for certain parts of the root tip, notably in the 5–10 mm segment, where some R. Q. values are as high as 1.5. It would be of interest to determine if these R. Q. values decrease at pH's higher than 4.5.

METHODS

Onion sets were sprouted in the manner previously described (Norris, '51), with the exception that micro-elements and iron were added to the one-quarter strength Hoagland's solution. This growth medium was maintained at a constant temperature of 25 ± 1°C.

When the roots were two days old, they were used for experimentation. The first three 5 mm segments, i.e. the 0–5, 5–10, and 10–15 mm segments, measuring from the apex of the root toward the bulb were employed. These segments were obtained by cutting the roots with small dissecting scissors to which a 5 mm pointer had been attached. The segments were collected in filter paper lined funnels, maintained in the growth medium still at 25°C. In all cases, samples of 100 mg (wet weight) were used.

The samples were transferred immediately to Warburg flasks containing 3 ml of M/15 phosphate buffer of the desired pH. Respiratory measurements, employing the Warburg constant volume respirometer, were made in an atmosphere of pure oxygen at 30°C. This was necessary, as it has previously been shown by Berry and Norris ('49) that the oxygen content of air limits the rate of respiration of the apical segment at this temperature. CO_2 output was determined by the "direct" method of Warburg employing three reaction vessels. Buffers with a pH higher than 5.0 bind appreciable amounts of CO_2, and this bound gas was released at the end of the one hour experimental period by tipping in an excess of acid from the side arm of the vessel.

Oxygen consumption was measured in the usual manner by adding 0.2 ml of 10% KOH to act as a CO_2 absorbent, to the center well of one of the triplicate experimental vessels. Results are reported as cubic millimeters of oxygen consumed or carbon dioxide produced per 100 mg (wet weight) of tissue per hour. The values shown in the tables are averages of the number of experimental measurements shown in the second column. Standard deviations are recorded.

TABLE 1

Effect of pH on gas exchange of the onion root tip

pH	NUMBER OF RUNS AVERAGED	O_2 UPTAKE mm³/100 mg wet weight of tissue/hr.	CO_2 EVOLVED mm³/100 mg wet weight of tissue/hr.	R. Q. RESPIRATORY QUOTIENT
		0 – 5 MM SEGMENT		
4.5	4	126 ± 6.3	138 ± 11	1.10
5.3	6	120 ± 9.9	127.5 ± 15	1.06
6.2	4	127.5 ± 3.2	118.8 ± 9.6	0.93
7.0	6	120 ± 7.6	102 ± 5.7	0.85
		5 – 10 MM SEGMENT		
4.5	5	37.3 ± 5.6	57.3 ± 6.8	1.53
5.3	7	37.2 ± 9.4	40.9 ± 7.9	1.10
6.2	5	36 ± 8.1	40 ± 7.6	1.11
7.0	10	34.5 ± 2.7	33.9 ± 8	0.98
		10 – 15 MM SEGMENT		
4.5	5	35.4 ± 2.8	45 ± 6.9	1.27
5.3	4	34.8 ± 8.4	41.3 ± 10.4	1.19
6.2	4	38.9 ± 3.5	35.4 ± 4.2	0.91
7.0	5	33.5 ± 1.8	25.7 ± 1.8	0.77

RESULTS

In table 1 there is recorded the effect of pH of the suspending buffer on gas exchange of the first three 5 mm segments of the onion root tip. Looking first at the 0–5 mm segment it is noted that the oxygen uptake is essentially the same at all of the pH values employed, however, there is a definite decrease in the quantity of carbon dioxide produced at the higher pH values. This results in a decrease in the respiratory quotient (last column of the table) with each increase in the pH (first column of the table) of the buffer in which the root segments were suspended.

Observing next the data for the 5–10 and 10–15 mm segments it is apparent that the results are similar to those shown for the 0–5 mm segment. That is, the oxygen uptake is essentially constant over the pH range employed, while CO_2 evolution falls rapidly with decreasing acidity, resulting in lowered respiratory quotients at the higher pH values. The values given by the 5–10 mm segment for pH's of 5.3 and 6.2 prove to be an exception; for which no explanation is readily available.

In general these results show precisely the same phenomenon for the onion root as was observed by Bonner and Wildman ('46) for spinach leaves and by Bonner ('34) for the avena coleoptile.

DISCUSSION

The magnitude of oxygen consumption shown by the root segments is in excellent agreement with results previously obtained over a period of several years in this laboratory, Norris ('56). Good agreement is also shown when the values recorded here are compared to previously reported values by Norris ('51) and by Berry and Norris ('49). It may be noted that the value for the intermediate segment (5–10 mm) is somewhat less in this study than that previously reported by Berry and Norris ('49).

With regard to oxygen consumption only, the current results are in agreement with the work of Jeffrey ('51). She found no change in rate of oxygen consumption when roots were suspended in buffers of pH values between 3.0 and 9.6. It should be mentioned however, that she used 30 mm apical segments of three-day-old onion roots and that she measured only oxygen consumption.

This investigation in conjunction with results previously obtained by Bonner ('34) and by Bonner and Wildman ('46) warn against evaluating the effect of the pH of suspending buffer on respiration in terms of one component of respiration only. That is, the overall effect may be quite different if both oxygen consumption and carbon dioxide production are measured, from the effect noted if only O_2 uptake or CO_2 output is determined.

In this work no effort was made to evaluate the influence of individual ions upon respiration of the root tips. This will be the object of a later report.

SUMMARY

1. Oxygen consumption and carbon dioxide production were measured on the first three 5 mm segments of the onion root tip using the Warburg manometric technique. These measurements were made when the pH value of the phosphate buffer in which the roots were suspended was 4.5, 5.3, 6.2, and 7.0.

2. Over this limited pH range there appeared to be no significant difference in oxygen uptake, however the carbon dioxide evolution decreased as the buffer became less acid. This results in a decreasing respiratory quotient as the pH increases from 4.5 to 7.0.

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THE EXCHANGE OF POTASSIUM IN FROG NERVE: ROLE OF AEROBIC METABOLISM AND GLYCOLYSIS

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THREE FIGURES

INTRODUCTION

The exchange of intracellular potassium for its labelled isotope has been studied in various systems by a number of investigators. The exchange process requires energy derived from metabolism, since anoxia decreases the exchange rate in pig kidney cortex slices (Whittam and Davies, '54) and reduces the specific activity of liver mitochondria incubated in a medium containing K^{42} (Stanbury and Mudge, '53).

Likewise, a decreased metabolic rate as brought about by lowering the temperature reduces the exchangeability of cell potassium for K^{42} from the medium. This was demonstrated in kidney cortex slices by Whittam and Davies ('53) and also by Bartley, Davies and Krebs ('54). Rabbit cerebral cortex slices (Aebi, '53), frog muscle (Harris, '52) and rabbit nerve (McLennan and Harris, '54) exhibit the same inhibition of potassium exchange at low temperature.

The experiments reported here have been undertaken to study the role of aerobic metabolism and of glycolysis in the exchange of potassium in frog nerves. The radioactive isotopes of potassium and rubidium, K^{42} and Rb^{86} have been used as tracers.

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The results obtained indicate that anaerobic conditions or DNP poisoning decrease the exchangeability of nerve potassium for K^{42} and Rb^{86}.

An increased glycolytic rate, as brought about by addition of glucose or arsenate to cyanide-poisoned nerves partially prevents potassium loss due to anoxia but does not favor a greater rate of exchange between nerve potassium and the labelled isotope.

The effect of metabolic inhibitions and of glycolytic rate on K fluxes has also been investigated.

METHODS

A. Storage and equilibration

Paired sciatic nerves from Rana pipiens were dissected and stored at 4°C. for about 12 hours in Ringer's solution containing (in m mole/liter): NaCl: 110, KCl: 2.0, $CaCl_2$: 1.08, $NaH_2PO_4 \cdot H_2O$: 1.33, Na_2HPO_4: 5.35.

Usually 4 pairs of frog nerves were used per experiment in order to obtain enough tissue for counting and total potassium determinations. Before starting equilibration, the nerves were allowed to stand at room temperature for one hour. Each set of nerves was then blotted on filter paper, weighed and transferred to flasks containing Ringer's labelled with the isotope under study and the metabolic inhibitor. The temperature was maintained at 18°C by immersing the flasks in a temperature-controlled water bath. The solutions were kept agitated by gently bubbling air through them. The pH of all solutions used was maintained between 7.25 and 7.40.

B. Radioactive tracers

All tracers used were purchased from the Union Carbide and Carbon Co., Oak Ridge, Tennessee.

Rubidium 86 (half-life: 19.2 days) obtained as $Rb^{86}{}_2CO_3$ was first converted to the chloride by addition of dilute HCl. $Rb^{86}Cl$ was weighed to prepare a 100 × concentrated stock solution. Ringer's solution with Rb^{86} as a tracer contained the same amounts of NaCl, $CaCl_2$ and phosphates as inactive Ringer's, but KCl was replaced by $Rb^{86}Cl$ (1.9 mM/l). Potassium 42 (half-life 12.44 hours) was weighed as $K^{42}{}_2CO_3$ and converted to $K^{42}Cl$ by addition of dilute HCl. The $K^{42}Cl$ stock solution contained 800 mM/l. K^{42} Ringer's had part or the whole of its KCl replaced by the tagged element, to obtain a total concentration of 2 mM/l.

C. Total potassium determinations

At the end of the experiments, the nerves were blotted on filter paper, weighed and placed in 100 ml Kjeldahl flasks and ashed in nitroperchloric mixture.

After cooling, the residue was transferred quantitatively to 25 ml graduated flasks. This solution was used for potassium determinations with a flame photometer.

In some Rb^{86} experiments, total radioactivity of the ashed nerves was determined by transferring 0.2 to 1 ml of the above solution to aluminum planchets, drying under an infrared lamp and counting with the Geiger-Müller counter.

Nerve K and Rb contents have always been expressed in terms of mM/kg whole nerve, on the basis of the initial weight and without correction for extracellular space.

D. Counting procedures

Two types of counters were used: (1) a well-type scintillation counter using a NaI crystal, after the design of Anger ('51). At given time intervals, the control and test nerves were transferred, after a quick washing in inactive solutions, to a small test tube fitting in the well of the counter. The uptake of radioactivity could thus be followed by direct tissue counting. The loss of activity from the nerves after transfer to the corresponding inactive solutions was followed in time according to the same procedure. (2) In some cases the loss of activity into inactive solutions was measured using a Geiger-Müller counter. The nerves were equilibrated for a given length of time in 2 ml of inactive solution from which 1 ml was dried on planchets before counting. This method has always been used simultaneously with direct tissue counting, either for measuring activity loss shortly after transfer to inactive solutions, or several hours after transfer when the radioactivity remaining in the tissue was too low to be measured directly.

E. Interpretation of results

The amount of tracer present in the nerves was calculated from the radioactivity measurements made during the experiment and compared to a standard. The uptake of Rb^{86} and K^{42} was thus followed up to 16 hours after starting equilibration in radioactive Ringer's. After 12 to 16 hours, both test and control nerves were transferred to inactive solutions and the loss of radioactivity was followed similarly. The results have been plotted as *total nerve activity* (mM tracer/kg whole nerve) as a function of time (hours).

From the "total activity curves" the following data have been calculated:

1. The rate of K^{42} or Rb^{86} uptake or loss, expressed in terms of mM/kg whole nerve/hour.

2. The percentage of total nerve K replaced by K^{42} after t hours equilibration in radioactive Ringer's:

$$\%\ \text{nerve K replaced by } K^{42} \text{ at time } t = \frac{(K^{42}\ \text{content})_t}{(\text{total K})_t} \times 100$$

The total K content of the tissues at time t was computed from the flame photometer data obtained at the end of the experiment, assuming a linear K loss due to metabolic inhibition.

The rate of total nerve K loss was estimated by comparison between the values obtained for the inhibited nerves and the controls without inhibitor.

In the case of Rb^{86}:

$$\%\ \text{nerve K replaced by } Rb^{86} \text{ at time } t = \frac{(Rb^{86}\ \text{content})_t}{(\text{total K} + Rb^{86})_t} \times 100$$

RESULTS

I. The exchange of nerve potassium for Rb^{86} and K^{42}

The total activity curves (such as figures 1 and 2) and the calculated percentage of total nerve potassium replaced by Rb^{86} after several hours equilibration in Rb^{86} Ringer's (Table 1) show that exchange is far from being complete even after 16 hours.

Flame photometer data are lacking for K^{42} experiments. However, assuming a total potassium concentration of 48 mM/kg whole nerve, no more than 27% was replaced by K^{42} after 12 hours equilibration in K^{42} Ringer's.

TABLE 1

Replacement of nerve potassium for Rb^{86}

NUMBER OF EXPERIMENTS	EQUILIBRATION TIME	Rb^{86} CONTENT (mM/kg whole nerve)	TOTAL K (mM/kg whole nerve)	% TOTAL K REPLACED BY Rb^{86}
	(hours)			
7*	2	2.65	43.25	6.1
2*	4	3.46	38.46	9.0
3°	7	6.60	45.30	14.6
3°	12	9.65	46.95	20.6
3*	16	10.50	44.00	23.9
	* = Winter frogs		° = Summer frogs	

The values given are averages for the number of experiments indicated.

The 7- and 12-hour values have been obtained from direct tissue counting (scintillation counter). All other values from ashed nerves (G.M. counter).

The rate of K^{42} and Rb^{86} uptake becomes approximately constant after two hours incubation in radioactive Ringer's. The rate of loss after transfer to inactive solutions obeys the same law and seems to be independent of the initial tracer's content of the nerves.

II. The effect of 2,4-dinitrophenol on the kinetics of potassium exchange

All experiments were done using 1×10^{-4} M DNP/l, at which concentration phosphorylations are uncoupled from respiration (Loomis and Lipman, '48). All solutions contained 5×10^{-3} M glucose/l.

A. Total activity curves. Analysis of figures 1 and 2 shows that DNP causes a marked decrease in the uptake of Rb^{86} or K^{42} from the outside medium. An experiment was performed to test the latency of the DNP effect on Rb^{86} uptake after addition to Rb^{86} Ringer's: the uptake dropped within half an hour.

B. Effect on total potassium content. Nerves exposed to DNP lose potassium and gain sodium slightly in excess of the

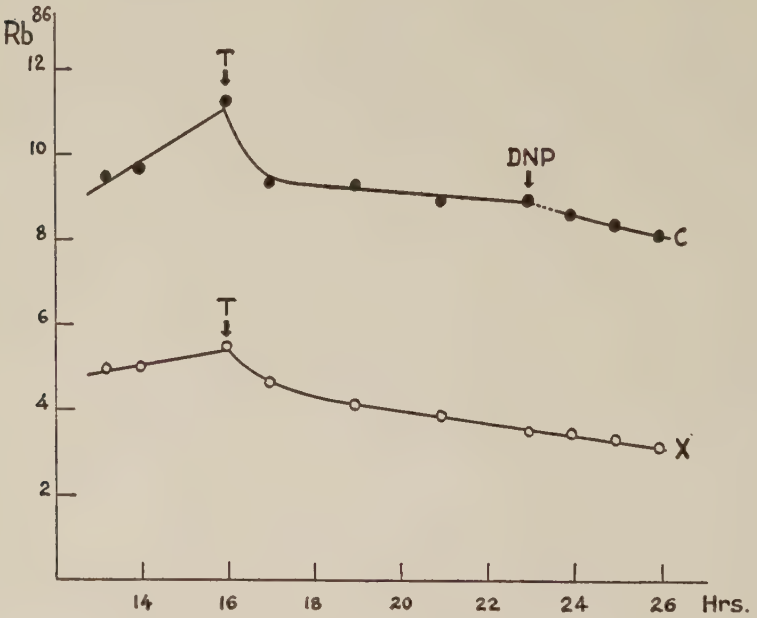

Fig. 1 The effect of 2,4-dinitrophenol on Rb^{86} uptake and loss by frog nerves equilibrated in Rb^{86} Ringer's.

C: controls in Ringer's without inhibitor.

X: Nerves in Ringer's + 1×10^{-4} M DNP.

The nerves were equilibrated for 16 hours in Rb^{86} Ringer's and the uptake followed for the last three hours.

At T: Transfer to inactive solutions.

At second arrow: Control nerves were transferred to inactive Ringer's containing 1×10^{-4} M DNP.

Ordinates: Total nerve activity expressed as mM Rb^{86}/kg whole nerve.

potassium loss. The rate of potassium loss varies between 0.44 and 1.1 mM/kg/hr (6 experiments) with an average of 0.9 mM/kg/hr.

C. Effect on the exchangeability of nerve potassium for Rb^{86}. The percentage of total nerve potassium replaced by Rb^{86} from the medium is reduced under the influence of DNP. After 7 hours equilibration in Rb^{86} Ringer's, poisoned nerves have exchanged 12% of their total potassium for the tracer, as compared to 15% in the controls (average of two experiments).

In some cases, where the loss of activity in inactive solutions was followed after equilibration in Rb^{86} Ringer's, the rate of potassium loss by the poisoned nerves is assumed

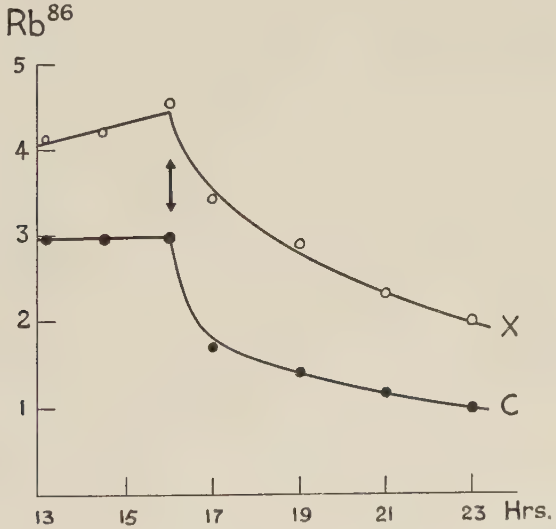

Fig. 2 The effect of arsenate and glucose on Rb^{86} uptake and loss by cyanide-poisoned nerves.

The nerves were equilibrated for 16 hours in Rb^{86} Ringer's and the uptake was followed for the last 3 hours:

C in Ringer's $+ 1 \times 10^{-3}$ M NaCN
X in Ringer's $+ 1 \times 10^{-3}$ M NaCN
$+ 5 \times 10^{-3}$ M glucose
$+ 5 \times 10^{-3}$ M $NaHAsO_4$

At the arrow, the nerves were transferred to inactive Ringer's containing the same inhibitors and substrate as above.

Ordinate: total nerve activity (mM Rb^{86}/Kg whole nerve).

to have occurred linearly and was computed from the flame photometer values obtained at the end of the experiment. Table 2 exemplifies data obtained in this fashion.

D. Effect on the rate of K^{42} and Rb^{86} uptake. The rates of K^{42} and Rb^{86} uptake have been calculated from the total activity curves obtained experimentally. In all cases DNP has an inhibitory effect.

TABLE 2

Effect of DNP on the exchangeability of nerve K for Rb^{86}

TIME	1 × 10^{-4}M DNP			CONTROL		
	Total K + Rb^{86} (mM/kg)	Rb^{86} (mM/kg)	% Total K replaced by Rb^{86}	Total K + Rb^{86} (mM/kg)	Rb^{86} (mM/kg)	% Total K replaced by Rb^{86}
(hours)						
			Uptake from Rb^{86} Ringer's			
13	38.62	4.85	12.5	49.6	9.20	18.5
14	37.78	5.05	13.0	49.6	9.80	19.75
15	36.94	5.25	14.2	49.6	10.40	21.0
16	36.10	5.45	15.1	49.6	11.00	22.2
			Loss to inactive solutions			
1	35.26	4.65	13.2	49.6	9.45	19.0
2	34.42	4.30	12.5	49.6	9.20	18.5
3	33.58	4.15	12.4	49.6	9.10	18.3
4	32.74	4.00	12.2	49.6	9.04	18.2
5	31.90	3.85	12.0	49.6	8.95	18.1
6	31.06	3.67	11.8	49.6	8.86	17.85
7	30.22*	3.50	11.6	49.6*	8.76	17.65

Nerves equilibrated for 16 hours in Rb^{86} Ringer's then transferred to inactive solutions.

Total K content computed for the DPN-poisoned nerves from the flame photometer data obtained at the end of the experiment (*). Rate of K loss: 0.84 mM/kg/hr.

DNP increases the rate of tracer loss after transfer to inactive solutions. This effect was observed also when DNP was added 5 hours (K^{42} experiments) or 7 hours (Rb^{86} experiments) after the transfer of control nerves to inactive Ringer's. The rate of tracer's loss increased soon after addition of DNP (table 3 and fig. 1).

III. The effect of cyanide on the kinetics of potassium exchange

The effect of cyanide on Rb^{86} uptake is very similar to that of DNP:

1. Potassium is lost from cyanide-poisoned nerves at a rate of 1.19 mM/kg/hr (average value of 4 experiments).

2. The percentage of total nerve potassium replaced by Rb^{86} is decreased under the influence of cyanide.

TABLE 3

The effect of DNP on the rate of tracer uptake and loss

EXPERIMENT NUMBER	TRACER	RATE OF UPTAKE		RATE OF LOSS	
		(mM/kg/hr) 1×10^{-4}M DNP	Control	(mM/kg/hr) 1×10^{-4}M DNP	Control
1	Rb^{86}	0.461	0.804		
2	Rb^{86}	0.228	0.498		
3	Rb^{86}	0.180	0.649	0.130	0.072
4	K^{42}	0.100	0.900	0.300	0.190
5	K^{42}	0.060	0.796	0.370	0.370

The rates of uptake have been calculated from the total activity curves after 10 hours equilibration in radioactive Ringer's; the rates of loss 5 hours after transfer of inactive solutions.

DNP was added to the control nerves 7 (experiment #3) and 5 hours (experiment #4 and #5) after transfer to inactive solutions. The rates of tracer loss increased as follows:

	RATE BEFORE DNP	RATE AFTER DNP
#3	0.072 mM/kg/hr	0.130 mM/kg/hr
#4	0.190	0.700
#5	0.370	0.400

After 16 hours equilibration in Rb^{86} Ringer's, 15% of the total potassium remaining in cyanide-poisoned nerves has been replaced by Rb^{86}, as compared to 20.5% in the controls (average value of three experiments).

3. The rates of uptake are markedly reduced in cyanide-poisoned nerves, but rates of loss are not affected. The results of a typical experiment are summarized in table 4.

Experiments have been done to test the effect of different cyanide concentrations on Rb^{86} uptake. Inhibition does not occur at 2×10^{-4} M/l while 1×10^{-3} M/l and 1×10^{-2} M/l produce the same effect.

We may here refer to the data obtained by Schmitt on nerve respiration ('31), which showed that the inhibition obtained using 1×10^{-3} M NaCN/l could not be further increased by using higher cyanide concentrations.

TABLE 4

The effect of NaCN on the rate of tracer uptake and loss
(Experiment #7)

TIME	RATE OF UPTAKE (mM/kg/hr)		TIME	RATE OF LOSS (mM/kg/hr)	
	1×10^{-3}M NaCN	Control		1×10^{-3}M NaCN	Control
(hours)			*(hours)*		
12	0.0450	0.655	0.5	0.859	1.020
13	0.0450	0.655	1.0	0.701	0.540
14	0.0450	0.655	1.5	0.318	0.258
15	0.0450	0.655	2.0	0.318	0.222
16	0.0450	0.655	2.5	0.300	0.162
			3.0	0.282	0.150
			3.5	0.144	0.150
			4.0	0.144	0.150
			5.0	0.144	0.150
			7.0	0.144	0.150

Nerves equilibrated in Rb^{86} Ringer's for 16 hours. The uptake of Rb^{86} was followed for the last four hours. After transfer to inactive solutions, the loss of activity was followed for 7 hours.

IV. The effect of glucose and arsenate on the K^{42} and Rb^{86} uptake by anoxic and cyanide-poisoned nerves

A. Anoxic nerves. After dissection, equilibration in the cold for 12 hours and weighing, both test and control nerves were placed in Erlenmeyer flasks containing phosphate-free Rb^{86} Ringer's. To the test nerves had been added 5×10^{-3} M/l Na_2HAsO_4. The N_2 used was first purified on hot copper in order to eliminate traces of oxygen, then bubbled through the solutions for half an hour. The flasks were sealed and put in a temperature-controlled water bath at 18°C for 12 hours. After weighing, ashing and dilution of the residue to a known volume, aliquots were counted with the G.M. counter. Total potassium content was determined by flame photometry.

The results are summarized in table 5. It follows that:

1. Arsenate prevents partly the potassium loss due to anoxia.
2. Rb^{86} uptake is greater in anoxic, arsenate-poisoned nerves than for the controls in nitrogen.
3. The percentage of total potassium replaced by Rb^{86} is not significantly altered.

TABLE 5

Effect of arsenate on anoxic nerves

EXPERIMENT	$N_2 + 5 \times 10^{-3}$ M Na_2HAsO_4			N_2		
	Rb^{86} (mM/kg)	Total K + Rb^{86} (mM/kg)	% Total K replaced by Rb^{86}	Rb^{86} (mM/kg)	Total K + Rb^{86} (mM/kg)	% Total K replaced by Rb^{86}
A-1	7.82	41.22	19.0	4.69	23.49	20.0
A-2	8.31	33.31	25.0	6.00	31.80	18.9
A-3	5.74	25.74	22.2	4.25	20.25	21.0
Average	7.29	36.49	20.5	4.98	25.18	19.8

Nerves equilibrated for 12 hours in phosphate-free Ringer's. Total potassium content obtained by flame photometry. The Rb^{86} content has been determined from the ashed tissues with the G.M. counter.

B. *Cyanide-poisoned nerves*

The effects of arsenate and glucose on the electrolyte balance and Rb^{86} or K^{42} uptake by cyanide-poisoned nerves has been investigated by adding 5×10^{-3} M/l Na_2HAsO_4 and from 2 to 5×10^{-3} M/l glucose to phosphate-free Ringer's already containing 1×10^{-3} M/l NaCN. In some cases, only glucose was added to phosphate-buffered Ringer's containing NaCN. Control nerves were equilibrated in Ringer's with cyanide only.

Uptake and loss of radioactivity was measured by direct tissue counting using the scintillation counter.

From analysis of the total activity curve, such as figure 3, we see that the uptake of Rb^{86} or K^{42} is greater in the presence of arsenate and glucose. Flame photometer data show a decreased rate of potassium loss but the percentage of nerve potassium replaced by the tagged ion is not significantly altered (table 6).

Arsenate and glucose, on the other hand, increase markedly the rates of tracer uptake and loss (table 7). Since the same phenomenon can be observed in the presence of glucose alone in cyanide-poisoned nerves (experiments A-12 and A-13, table 7) it seems that its cause must be attributed to the increased rate of glycolysis induced by either glucose or arsenate or both.

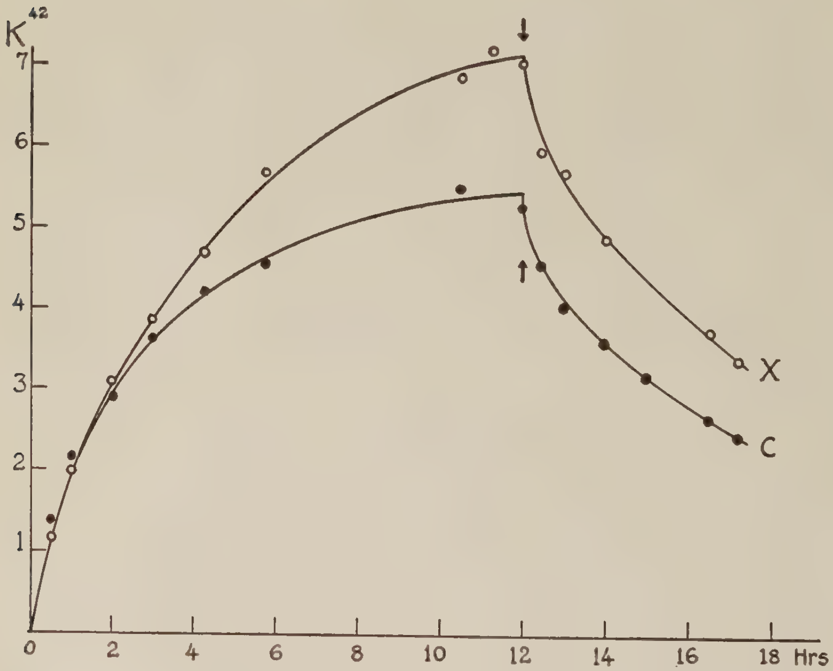

Fig. 3 The effect of arsenate and glucose on K^{42} uptake and loss by cyanide-poisoned nerves.

C nerves were equilibrated for 12 hours in K^{42} Ringer's containing 1×10^{-3} M NaCN. X nerves in K^{42} Ringer's with 1×10^{-3} M. NaCN + 5×10^{-3} M glucose + 5×10^{-3} M $NaHAsO_4$.

At arrow: transfer to inactive Ringer's containing the same inhibitors and substrates as above.

Ordinate: total nerve activity (mM K^{42}/Kg whole nerve).

V. Comparison between the results obtained with K^{42} and Rb^{86}

From the tables and figures given here the similarity between the results obtained with K^{42} and Rb^{86} is quite obvious. Uptake curves obtained under similar experimental conditions are comparable. Metabolic inhibitors affect fluxes and exchangeability of nerve potassium for K^{42} and Rb^{86} in a similar fashion.

TABLE 6

Effect of glucose and arsenate on the exchangeability of nerve K for K^{42} or Rb^{86}

EXPERIMENT	TRACER	1×10^{-3}M NaCN + 5×10^{-3}M Na_2HAsO_4 + 5×10^{-3}M glucose			1×10^{-3}M NaCN + 5×10^{-3}M Na_2HPO_4		
		Rb^{86} or K^{42} content (mM/kg)	Total K (mM/kg) + Rb^{86}	% K replaced by tracer	Rb^{86} or K^{42} content (mM/kg)	Total K (mM/kg) + Rb^{86}	% K replaced by tracer
A-4	Rb^{86}	5.7	37.6	15.2	4.1	31.1	16.4
A-5	Rb^{86}	5.1	35.3	14.4	3.8	24.2	15.7
A-6	Rb^{86}	2.0	13.5	14.8	1.8	10.1	17.7
A-7	Rb^{86}	2.0	11.3	17.7	1.7	9.3	18.3
A-10	K^{42}	2.61	23.6	11.0	1.49	15.9	9.4
A-11	K^{42}	3.0	23.75	12.6	2.12	16.27	13.6

Experiments A-4 and A-5: Values after 9 hours equilibration in Rb^{86} Ringer's.

Experiments A-6 and A-7: Values after 25 hours equilibration (17 hours in Rb^{86} Ringer's followed by 8 hours in inactive solutions).

Experiments A-10 and A-11: Values after 18 hours equilibration (12 hours in K^{42} Ringer's followed by 6 hours in inactive solutions).

Since Rb^{86} and K^{42} experiments have been carried over different lengths of time, comparison between exchangeability of nerve K for the two tracers is difficult to make. However, examination of tables 3 and 7 shows that rates of uptake and loss are usually greater for K^{42} than for Rb^{86}.

But these differences are only quantitative. On the whole, we feel that the long half-life Rb^{86} can be advantageously used instead of K^{42} provided experiments are also done with the latter to point out the quantitative differences between their physiological behavior.

TABLE 7

The effect of glucose and arsenate on the rate of tracer uptake and loss

EXPERIMENT	TRACER	RATE OF UPTAKE (mM/kg/hr)		RATE OF LOSS (mM/kg/hr)	
		1×10^{-3} M NaCN + 5×10^{-3} M Na_2HAsO_4 + 5×10^{-3} M glucose	1×10^{-3} M NaCN	1×10^{-3} M NaCN + 5×10^{-3} M Na_2HAsO_4 + 5×10^{-3} M glucose	1×10^{-3} M NaCN
A-4	Rb^{86}	0.288	0.0395		
A-5	Rb^{86}	0.135	0.0432		
A-6	Rb^{86}			0.150	0.096
A-7	Rb^{86}			0.132	0.102
A-8	Rb^{86}	0.150	0	0.210	0.102
A-9	Rb^{86}	0.162	0	0.186	0.114
A-10	K^{42}	0.198	0	0.402	0.348
A-11	K^{42}	0.126	0	0.420	0.318
A-12	K^{42}	0.060	0	0.246	0.198
A-13	K^{42}	0.180	0	0.390	0.240

Rates of uptake have been calculated from the total activity curves after 10 hours equilibration in radioactive Ringer's; rates of loss 5 hours after transfer to inactive solutions.

The test nerves in experiments A-12 and A-13 were equilibrated in Ringer's containing NaCN and glucose but no arsenate.

DISCUSSION

Potassium balance and exchange are similarly affected by cyanide and 2,4-dinitrophenol. Since DNP, at the concentration used in these experiments, has been shown to uncouple phosphorylations from oxidations, we can conclude that availability of high-energy phosphate bonds is necessary for the maintenance of ionic equilibrium.

In control nerves without inhibitors, the process of K exchange is slow. After 16 hours equilibration in radioactive Ringer's only 25% of the total nerve K has been replaced by the tracer from the medium. No experiments have been done to test the extent of K exchange over longer periods of time.

Inhibition of aerobic sources of energy decrease the replaceability of nerve K for its labelled isotope to an appreciable extent.

The rate of K^{42} or Rb^{86} uptake from the medium depends on aerobic energy sources. On the other hand, the rate of transfer of the labelled K from nerves to medium does not depend on the internal concentration since it proceeds at approximately the same rate in control nerves as in NaCN-poisoned tissue.

This apparent independency of outward rates of K^{42} or Rb^{86} transfer whatever the metabolic state of the cells is probably secondary to other factors.

After transfer to inactive solutions, control nerves take up inactive K, from the medium, at the same rate as the K^* uptake during equilibration in radioactive Ringer's. This amounts to an average of 0.7 mM K/kg/hr which "dilutes" the K^{42} or Rb^{86} present in the exchangeable K fraction of the nerves, thus leading to a rate of radioactivity loss that is probably much lower than the actual rate of K exchange. Such "dilution" of labelled K cannot take place in metabolically inhibited nerves where uptake from the medium occurs at a very slow rate, together with a continuous loss of cell K.

The initial rate of K^{42} or Rb^{86} loss after transfer to inactive solutions seems to occur much faster in control nerves since no time has been allowed for dilution to become apparent.

The protection offered by glucose against anoxic potassium loss which was also demonstrated in frog nerves (Shanes, '52) and brain cortex slices (Dixon, '49), lead us to investigate whether or not this effect was linked to phosphorylations from the glycolytic cycle. Since arsenate increases fermentation in yeast (Meyerhof *et al.,* '37) and lactic acid production in muscle (Needham and Pillai, '37) but uncouples phosphorylations from glycolysis, the possible role of high-energy phosphate bonds in the "protection" offered by a high rate of glycolysis against anoxia could be investigated.

Our data on cyanide-poisoned nerves show that the same effects are observed whether the rate of glycolysis is increased by glucose and/or arsenate. Hence, the higher potassium concentration maintained in actively glycolyzing nerves cannot be ascribed to a greater availability of high-energy phosphate bonds.

For this reason, and also since glucose and arsenate do not restore, even partially, the ability of nerve K to exchange for its isotope, the "glucose protection" against cyanide-poisoning cannot easily be considered as a partial reversal of anoxic inhibition.

The rate of K* loss after transfer to inactive solutions occurs at a much faster rate in actively glycolysing nerves than in anoxic controls without glucose. In this case, this rate is proportional to the inside K concentration at any given time, calculated as:

$$\frac{\text{rate of K* loss (mM/kg/hr)}}{\text{K content (mM/kg)}} \text{ at time t}$$

after transfer to inactive solutions.

The calculated values for 5 hours after transfer to inactive solutions are mentioned in table 8.

The process of K exchange in frog nerves is of a complex nature. On the one hand, aerobic metabolism is necessary for maximum exchange. On the other, the rate of glycolysis in anoxic nerves protects against K loss, but the rates of K transfer in both directions are so increased as to keep the percentage of nerve K exchanged within a given time to the low anoxic value. The "glucose" protection does not affect all of nerve K, but only a small fraction of it. Within that fraction, the exchange process is a function of the inside K concentration.

TABLE 8

Relation between the rate of K^* loss and total K content in frog nerves

EXPERIMENT NUMBER	TRACER	INHIBITORS		$\frac{\text{RATE OF } K^* \text{ LOSS}}{\text{TOTAL K CONTENT}}$	
		X	C	X	C
3	Rb^{86}	1×10^{-4}M. DNP	none	.034	.012
4	K^{42}	1×10^{-4}M. DNP	none	.078	.017
5	K^{42}	1×10^{-4}M. DNP	none	.072	.016
6	Rb^{86}	1×10^{-2}M. NaCN	none	.062	.014
7	Rb^{86}	1×10^{-2}M. NaCN	none	.062	.046
A-6	Rb^{86}		1×10^{-3}M. NaCN	.060	.045
A-7	Rb^{86}		1×10^{-3}M. NaCN	.055	.049
A-8	Rb^{86}		1×10^{-3}M. NaCN	.087	.086
A-9	Rb^{86}	1×10^{-3}M. NaCN + 5×10^{-3}M. Na_2HAsO_4 + 5×10^{-3}M. glucose	1×10^{-3}M. NaCN	.065	.076
A-10	K^{42}		1×10^{-3}M. NaCN	.132	.191
A-11	K^{42}		1×10^{-3}M. NaCN	.122	.127
A-12	K^{42}		1×10^{-3}M. NaCN	.083	.095
A-13	K^{42}		1×10^{-3}M. NaCN	.103	.109

All figures correspond to rates of K^* loss and total K content 5 hours after transfer to inactive solutions.

The effect of DNP on K^* loss can perhaps be correlated with an inhibition of the Pasteur effect, as shown by Terner ('52) in brain slices. The increased rate of K^* loss in frog nerves would, in this case, be related to a higher rate of glucose breakdown induced by DNP. Experiments are now in progress to investigate this point.

Hodgkin and Keynes ('55) working with Loligo and Sepia axons have shown the absence of an effect of NaCN and DNP on K outfluxes. That DNP increases the outflux of K in frog nerves whereas it does not on Sepia axons could result from a different metabolic makeup of the tissues studied.

It is at present difficult to determine to what extent these different factors come into play for the maintenance of ionic equilibrium. But it seems possible that enzymatic reactions from the glycolytic cycle play a direct — if partial — role in K transfer across the nerve boundary.

SUMMARY

1. Frog nerves equilibrated in Ringer's where KCl has been replaced by Rb^{86} Cl (1.91 mM/l) exchange 20% of their K for Rb^{86} in 16 hours.

The uptake of Rb^{86} from Rb^{86} Ringer's or of K^{42} from Ringer's containing 2.0 mM/l $K^{42}Cl$ occurs in two phases: a fast initial uptake followed by a slower, linear gain.

2. 2,4-dinitrophenol at a concentration of 1×10^{-4} M/l inhibits Rb^{86} and K^{42} uptake and causes an average loss of nerve potassium amounting to 0.9 mM/kg/hr.

3. The percentage of nerve potassium replaced by Rb^{86} or K^{42} after several hours of equilibration in radioactive Ringer's is also decreased by DNP.

4. The rate of radioactivity loss from nerves transferred to inactive solutions after 12 to 16 hours equilibration in Rb^{86} or K^{42} Ringer's is increased by DNP.

5. NaCN, at a concentration of 1×10^{-3} M/l has the same effect as DNP. Rates of loss, in the absence of glucose, are not affected.

6. Glucose, when added to cyanide-poisoned nerves decreases the rate of potassium loss and increases the amount of Rb^{86} or K^{42} taken up in a given length of time. But the percentage of nerve potassium replaced by the tracer during the equilibration period in radioactive Ringer's is not affected by the presence of glucose.

7. The rate of activity loss from cyanide-poisoned nerves is greater in the presence of glucose.

8. The effect of glucose on cyanide-poisoned nerves is not affected by addition of 5×10^{-3} M Na_2HAsO_4/l.

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CONTRASTING INHIBITORY EFFECTS OF PROBENECID ON THE RENAL TUBULAR EXCRETION OF P-AMINOHIPPURATE AND ON THE ACTIVE REABSORPTION OF UREA IN THE DOGFISH, SQUALUS ACANTHIAS[1]

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Urea has in common with uric acid the feature of being excreted against concentration gradients by the renal tubules of certan vertebrates while being actively reabsorbed after glomerular filtration by the renal tubules of others. It is of interest to know what characteristics are shared by these cellular processes which transfer identical compounds in opposite directions.

Probenecid, or p-(dipropylsulfamyl)-benzoic acid, at concentrations which do not affect either glomerular filtration rates or cellular oxidative processes, depresses the tubular transport of uric acid in mammals, whether this transfer is in the direction from blood to urine as in the Dalmatian dog (Beyer, et al., '51) and in the rabbit (Poulsen, '55) or in the reverse direction as in man and various other mammals (Beyer, et al., '51). Similarly Probenecid specifically blocks the active tubular excretion of urea in bullfrogs where the cellular transfer process is characterized by a maximum rate (Tm) at high plasma urea concentrations (Forster, '54). Inasmuch as filtered urea is actively reabsorbed by dogfish and other Elasmobranchii (Smith, '36 and Kempton, '53) where

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its high concentration in body fluids plays an important role in osmotic regulation, it was of interest to test whether Probenecid acts similarly to depress the tubular transport of urea in these forms where net transfer is in the direction opposite to that in bullfrogs.

METHODS

The experiments were performed on male specimens of the dogfish, *Squalus acanthias.* The fish were caught on barbless hooks on hand lines in Frenchmans Bay. Specimens were rejected which showed extensive bleeding from the mouth wound. During the experiments urine was collected in rubber balloons attached to polyethylene catheters which were secured into the urogenital papillae. Blood samples were obtained by syringe from the caudal vessels.

p-Aminohippuric acid (PAH) and its acetylated derivative (PAAH) were determined by the method of Bratton and Marshall (39). Inulin clearances were used to evaluate glomerular filtration rates. Inulin concentrations in plasma and urine were measured by Roe's resorcinal method as modified by Schreiner ('50). Urea was determined by the microdiffusion method of Conway ('50).

Inulin and PAH were injected intramuscularly at least 12 hours before the start of urine and blood collections to provide for even distribution in body fluids and to obtain slowly falling concentrations in blood during the experimental clearance periods. Quantitative urine collections were made during four three-hour periods. Three blood samples were taken, one at the start of the first period, one at the end of the third period and one at the end of the experiment. Plasma values corresponding to the midpoints of the individual urine collections were obtained by interpolation from a semi-log plot.

RESULTS

The results obtained from experiments on two dogfish are shown in table 1. It is evident that a dose of Probenecid

TABLE 1

Effect of probenecid on the tubular excretion of PAH + PAAH and on the reabsorption of urea

TIME	URINE FLOW	INULIN CLEARANCE	PAH + PAAH			UREA			
			Plasma	Urine	Secreted	Plasma	Urine	Reabsorbed	
minutes	*ml/kg/day*	*ml/kg/day*	*mM/liter*	*mM/liter*	*mM/kg/day*	*mM/liter*	*mM/liter*	*mM/kg/day*	*mM per ml glomerular filtrate*
Dogfish no. 17 1.93 kg									
Inulin 3.3%, PAH 3.3%, 12 ml i.m. at — 16 hours									
0–225	46.2	178	1.39	13.00	.35	323	250	46.0	.26
225–350	48.5	163	1.24	11.60	.31	330	185	44.6	.27
350	100 mg Probenecid i.v.								
350–515	42.0	122	1.11	5.95	.12	325	175	32.3	.27
515–720	36.5	112	1.07	5.64	.09	310	140	29.6	.26
Dogfish no. 21 2.3 kg									
Inulin 3.3%, PAH 3.3%, 10 ml i.m. at — 18 hours									
0–215	33.6	116	.654	7.73	.18	364	80	39.5	.34
215–350	31.5	104	.567	7.50	.18	364	80	35.1	.34
350	100 mg Probenecid i.v.								
350–470	40.1	107	.526	3.94	.10	364	72	35.9	.34
470–620	31.3	99	.515	3.32	.05	364	54	34.3	.35

sufficient to depress the tubular secretion of PAH and PAAH by more than 50% was ineffective in altering the rate of urea reabsorption expressed as millimoles absorbed per unit volume of glomerular filtrate. The reabsorption of urea expressed in absolute amounts (mM/kg/day) showed a slight fall when there was a concomitant reduction in glomerular filtration rate after Probenecid, as in dogfish no. 17. That the reabsorption of urea varied with the glomerular filtration rate is not surprising in view of the fact that between 81 and 94% of the filtered urea was reabsorbed in these specimens. In dogfish no. 21 where glomerular filtration rates were relatively constant, the rate of urea reabsorption was unaffected by Probenecid administration, whether expressed as millimoles per kilogram body weight per day or as millimoles per milliliter of glomerular filtrate. Simultaneously the secretion of PAH plus PAAH was depressed by Probenecid to 30% of control values.

SUMMARY

Probenecid which blocks tubular excretion of urea in the frog is ineffective in depressing active reabsorption of urea by dogfish renal tubules with doses which reduce significantly the simultaneous transport rates of PAH and PAAH.

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SENSITIVITY OF UV-IRRADIATED BLEPHARISMA UNDULANS TO HIGH PRESSURE LYSIS

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ONE FIGURE

INTRODUCTION

UV-studies on single cells have yielded considerable information about some of the molecular components of the protoplasm and the role of these components in cellular metabolism (see Giese, '53). Likewise, hydrostatic pressure has provided a means for studying protoplasmic gel structure and for analyzing the functional significance of intracellular sol $\rightleftharpoons$ gel changes (see Marsland, '50; Marsland and Landau, '54; and Landau, Zimmerman and Marsland, '54). Accordingly, the present study, in which both UV-irradiation and high pressure have been employed, represents a first attempt to corelate observations from these two important areas.

Blepharisma undulans, the familiar red-pigmented heterotrichous ciliate, was used as the experimental organism. This animal can be cultured readily and it is not difficult to obtain large numbers derived from a single isolated individual. Moreover, preliminary experiments showed that blepharisma becomes structurally unstable, not only when it is exposed to high dosages (around 18,000 ergs/mm^2) of UV-irradiation, especially at certain wavelengths (see Hirshfield and Giese,

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'53), but also when it is subjected to high levels (10,000–12,000 lbs./in.^{2}) of hydrostatic compression. This structural instability is evidenced by a drastic and characteristic form of lysis. Numerous small blisters begin to form, first in the anterior tapered part of the organism, suggesting that the pellicle becomes detached from the subjacent cytoplasm. Then there is a rupturing of the surface layers in the anterior region, accompanied by an escape of granular material into the medium. Frequently the broad central part of the cytoplasm may become sealed off from the disintegrating anterior part, and regeneration may occur if the experimental treatment is not too prolonged or drastic. However, with more drastic treatments the blistering and rupturing are progressive. The lysis next involves the posterior tapered part of the organism and then the broad central part, so that finally, within 3–4 minutes, total lysis occurs.

This structural instability of the blepharisma, therefore, provided an experimental criterion by which the joint effects of both pressure and UV-irradiation could be evaluated.

MATERIALS AND METHODS

About sixty blepharisma, all derived from a single young "clone" (less than 11 days after isolation) were used for each experimental run. These young animals displayed a remarkably constant resistance to the pressure and irradiation effects, whereas "older" individuals became progressively much more sensitive.

Each group was washed thoroughly in Brandwein solution and then irradiated in a Perkin-Elmer Monochromator with a 250 watt high pressure mercury-argon lamp. The intensity of the monochromatic energy was measured by a photomultiplier (1-P-28) previously calibrated against a standard lamp. The animals were placed in shallow fused quartz depression slides and then irradiated from below.

Immediately after the irradiation, the animals were divided equally into a control group, which was maintained at atmospheric pressure, and an experimental group, which was subjected to the pressure treatment within 25 minutes. A second control group, consisting of about 30 non-irradiated animals from the same ''clone,'' was also exposed to equivalent levels of compression. The animals were examined carefully and counted accurately before and after each irradiation and immediately before each exposure to pressure. The pressurized specimens were under continuous observation and were counted in the pressure bomb, while the pressure was maintained.

The wavelengths used in the experiments were 230, 254, 265, 280, 302, 313, 334 and 365 mμ, respectively, and two dosages, namely 6000 and 12000 ergs/mm^2, were given in each case. Generally speaking these dosages, of themselves, proved to be sublytic, although in a few experiments a small percentage (less than 5%) of lysis was observed. Some experiments, utilizing 18,000 and 24,000 ergs/mm^2, were also attempted, but these dosages gave such high percentages of lysis that the samples could not be used for further study.

The experimental pressures ranged from 2,000–10,000 psi and all experiments were performed at 20°C. The pressure equipment was the same as that described by Marsland ('50). Pressure could be built up to any required level in a matter of about 10 seconds and the ''bomb'' is designed to permit continuous microscopic observation of the specimens throughout the compression period.

The critical lysis pressure was determined for each particular exposure and wavelength of UV-irradiation. This represents the minimum pressure at which 50% of the exposed organisms undergo total lysis within three minutes. At least five runs were made at each wavelength in order to determine very accurately the critical lysis pressure for that particular treatment.

OBSERVATIONS

The results of the experiments are presented in table I and figure 1. In this graph, the *reciprocal* of the critical lysis pressure is plotted against the wavelength. In essence, therefore, the graph presents two pressure-sensitivity curves, which can be regard as UV-action spectra with reference to the pressure effects.

The curves plainly show that the organism is more highly pressure-sensitive after irradiation in the protein absorbing region (280 mμ) than after irradiation in the nucleic acid absorbing region (265 mμ). However, the greatest sensitivity is displayed by organisms receiving the 230 mμ radiations, in which the absorption is much less specific.

TABLE 1

Effect of UV irradiation on the sensitivity of Blepharisma undulans to the lytic effects of high hydrostatic pressure

DOSAGE	WAVELENGTH	CRITICAL PRESSURE	RECIPROCAL PRESSURE
ergs/mm²	*mμ*	*psi*	
6,000	230	5,000	2.00×10^{-4}
	254	6,000	1.67×10^{-4}
	265	7,000	1.43×10^{-4}
	280	6,500	1.54×10^{-4}
	302	10,000	1.00×10^{-4}
	313	10,000	1.00×10^{-4}
	334	10,000	1.00×10^{-4}
	365	10,000	1.00×10^{-4}
	controls	10,000	1.00×10^{-4}
12,000	230	4,000	2.50×10^{-4}
	254	5,000	2.00×10^{-4}
	265	6,000	1.67×10^{-4}
	280	5,500	1.82×10^{-4}
	302	9,000	1.11×10^{-4}
	313	9,000	1.11×10^{-4}
	334	10,000	1.00×10^{-4}
	365	10,000	1.00×10^{-4}
	controls	10,000	1.00×10^{-4}

Several experiments were performed in which the pressure was raised by 1000 psi in a step-wise manner, allowing three minutes at each successive level, until a pressure resulting in a lysis of 50% of the organisms was achieved. This treatment seemed to "condition" the blepharisma so that the critical lysis pressures for both radiated and non-irradiated animals was consistently higher by about 1000 psi. In general, therefore, it may be stated that while this ''conditioning'' treatment raised the absolute values of critical lysis pressures, the differentials between irradiated and non-irradiated samples remained the same.

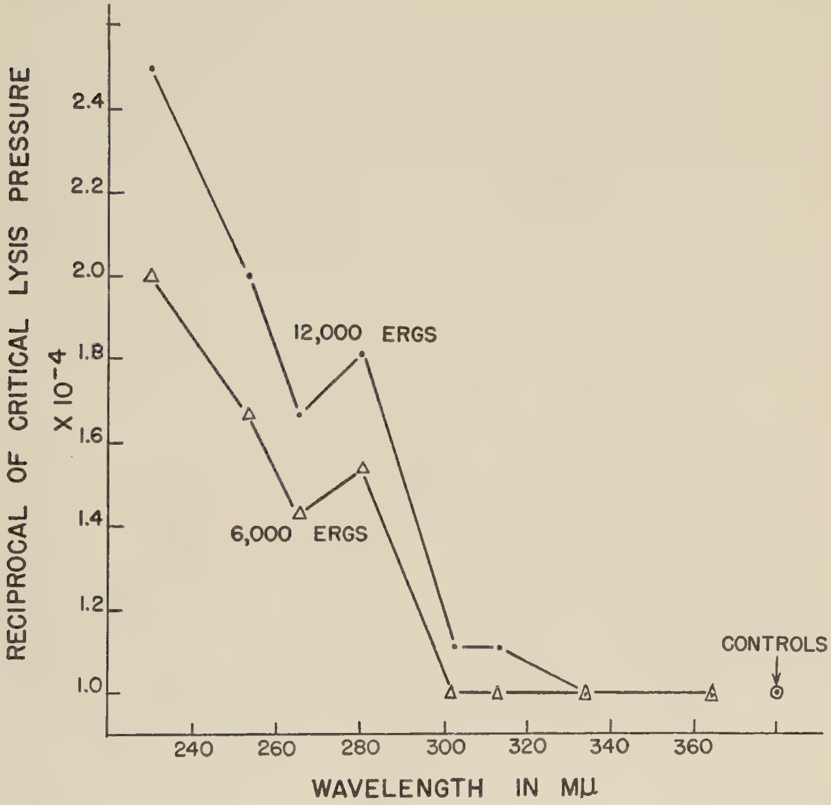

Figure 1

DISCUSSION

The data clearly show that a lowered resistance to pressure is most evident at low wavelengths (230 mμ) and in the protein-absorbing region (280 mμ). The 230 mμ effect corresponds well with previous observations (Giese, '53) which demonstrated that the organisms are immobilized for several hours by relatively low dosages (8,000 ergs/mm^2); whereas at higher wavelengths (265 mμ) immobilization occurs only with higher dosages (16,000 ergs) and is followed immediately by blistering and lysis. Probably the low wavelengths are acting primarily upon the cortical layers of the cell, as has been noted by Giese ('53). Indeed, Giese found that the pellicle and subjacent cytoplasm displayed drastic structural changes after such treatment. Also it seems generally agreed that these low wavelengths are absorbed rather indiscriminately by the various molecular components of the cell. Accordingly their depth of penetration is limited and their effects are mainly upon the superficial layers.

The other conspicuous peak of sensitivity, which occurs at 280 mμ, seems to represent a fairly specific effect upon the protein components of the system. However, the nucleic acid components may also be involved to some extent since there is a lesser extent of sensitivity at 265 mμ, a region where there is a heavy absorption by these components.

The precise nature of the lysis by high pressure and/or UV-irradiation is problematical. However, it seems likely that the phenomenon may involve the well-known solational effect of pressure upon protoplasmic gel structures (Marsland, '50). In most, if not all animal cells, the cortical cytoplasm, immediately subjacent to the plasma membrane and pellicle, displays an organized gel structure. It might be expected, therefore, that complete solation of this cortical cytoplasm would lead to molecular rearrangements which could very drastically alter the structure and the permeability characteristics of the cell. Moreover, such an expectation would seem particularly valid for highly organized cell types, such as blepharisma, which tend to maintain a fairly complex and elongate cell form.

It is generally agreed (see Kopac, '50) that the formation of a protoplasmic gel involves the unfolding of globular (protein) units into elongate fibrils which then become interlinked forming a three-dimensional colloidal network. Apparently one primary effect of pressure is to weaken the intermolecular linkages of the system (Marsland and Landau, '54), and a similar effect may be postulated for the UV-treatment. In fact, McLaren and Finkelstein ('50) and Giese and Crossman ('45) have shown that the disruption of chymotrypsin and albumen is clearly facilitated after appropriate UV-irradiation — which indicates an increase in the bond sensitivity. This interpretation implies that high pressure and UV-irradiation have additive effects because both agents produce a weakening of the intermolecular bonds of the gel system. However, it also seems possible that the UV-denaturation of a protein which normally participates in the formation of gel structure might be involved. This would result in the formation of a weaker gel, more susceptible to pressure solation. Such a view, indeed, is supported by the recent report of Giese ('53) that sublethal dosages of ultraviolet increase the susceptibility of paramecium to the killing effect of heat treatments, and that the action spectrum for this effect is that of a protein component.

Other studies (Hirshfield and Pecora, '55) on the effects of colchicine on blepharisma indicate that oriented protein molecules, which are so important in protoplasmic gelation, may also play a critical role in protozoan regeneration. Moreover, the present experiments seem to provide a basis for further UV-studies on cell division, since this phenomenon likewise appears to depend upon the maintainence of a definite cortical gel structure (Marsland and Landau, '54).

SUMMARY

UV-irradiated *Blepharisma undulans* were studied with reference to their sensitivity to lysis by high pressures in the range up to 10,000 lbs./in.^{2}. The wavelengths selected were 230, 254, 265, 280, 302, 313, 334 and 365 mμ; and two dosages, namely 6,000 ergs/mm^2 and 12,000 ergs/mm^2, were used at each wavelength. In each experiment the minimum pressure required to induce complete lysis in 50% of the

irradiated organsms was determined. This was designated as the critical lysis pressure.

The non-irradiated controls displayed a minimum sensitivity, the critical lysis pressure being 10,000 psi. Among the irradiated specimens, greatest sensitivity was evidenced at 230 mμ, slightly less at 280 and still less at 265, the critical lysis pressures (at 6,000 ergs) being 5,000, 6,500 and 7,000 psi, respectively. Other wavelengths had little or no sensitizing effect and at the effective wavelengths the sensitizing effect was directly related to the dosage.

Tentatively, these relative sensitivities to pressure are interpreted on the basis of the fairly specific UV-effects upon proteins (at 280 mμ) and nucleic acids (at 265 mμ) and of more generalized effects upon the cell cortex (at 230 mμ).

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PHOTODYNAMIC EFFECT OF BLEPHARISMA PIGMENT ON NERVE[1]

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ONE FIGURE

The pigment extracted from the ciliate *Blepharisma undulans* injures or kills marine eggs and colorless protozoans in the dark when present in high concentration. In low concentration it kills these forms only on exposure to intense visible light. The same pigment has no effect on the action potential of the entire frog sciatic nerve even when the nerve has been soaked in it for several hours and then exposed to intense visible light (Giese, '53). Since the nerve sheath acts as a diffusion barrier to many substances (Crescitelli, '51), the experiments were recently repeated after removing the sheath and separating the fibers. Experiments were also performed with nerves of the edible crab, *Cancer magister.* The positive results are reported below in addition to some performed with rose bengale for comparison.

The methods of handling the nerves were essentially like those used in the preceding paper (Pierce and Giese, '57), therefore they need not be described here, with the exception that a plastic perfusion chamber was employed, requiring the use of a Grass isolation unit. The visible light source was a GE-H100 SP-4 spotlamp which was set up with heat and ultraviolet filters as described in the preceding paper. The intensity at the level of the nerve was 2080 foot candles.

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[1] Supported in part by Contract AT(11-1)-234 with the U. S. Atomic Energy Commission. I am indebted to Mr. Stirling Pierce for help with the equipment and a number of the experiments, and to Dr. Theodore Bullock for the loan of a nerve perfusion chamber which served as a model for the one used.

Blepharismas, grown in the manner previously described (Giese, '49), were centrifuged at 10 × gravity in a hand centrifuge and washed in triply distilled water before they were again concentrated and ground into a pigment paste in a glass mill filled with the appropriate Ringer solution. By a high concentration of pigment is meant the pigment from 10^5 animals in 1 ml of Ringer solution. A high concentration had to be used in illumination experiments because the pigment itself is bleached by light and rapidly loses its potency (Giese, '53).

EXPERIMENTAL RESULTS

1. Effects of Blepharisma pigment. When a bundle of about 10 frog neurons was placed in the chamber flooded with Blepharisma pigment, in the dark it showed the same action potential as before adding the pigment and remained constant for 4 hours of observation. No coloring of the neurons was observed. The pigment therefore has no effect in the dark. A nerve bundle in Ringer's solution without pigment kept in the light of the spotlamp for several hours continued to discharge without more than a 5–8% drop; therefore, the visible light alone has no appreciable effect upon the nerve bundle.

When a frog nerve in Blepharisma pigment extract is also illuminated with intense visible light, the action potential falls to zero within a short time, in cases where only 1–4 fibers are present, almost immediately. When the pigment is diluted, the decline is slower and the pigment is bleached and loses effectiveness. The experiments were repeated many times on the nerve bundles of three bullfrogs with the same general results. The nerves were not observed to change color; apparently Blepharisma pigment is not absorbed in quantity.

Crab neurons in highly concentrated Blepharisma pigment in the dark gave a constant spike with the same threshold until the experiment was discontinued after two hours. No change in sensitivity of the neurons was shown by the strength

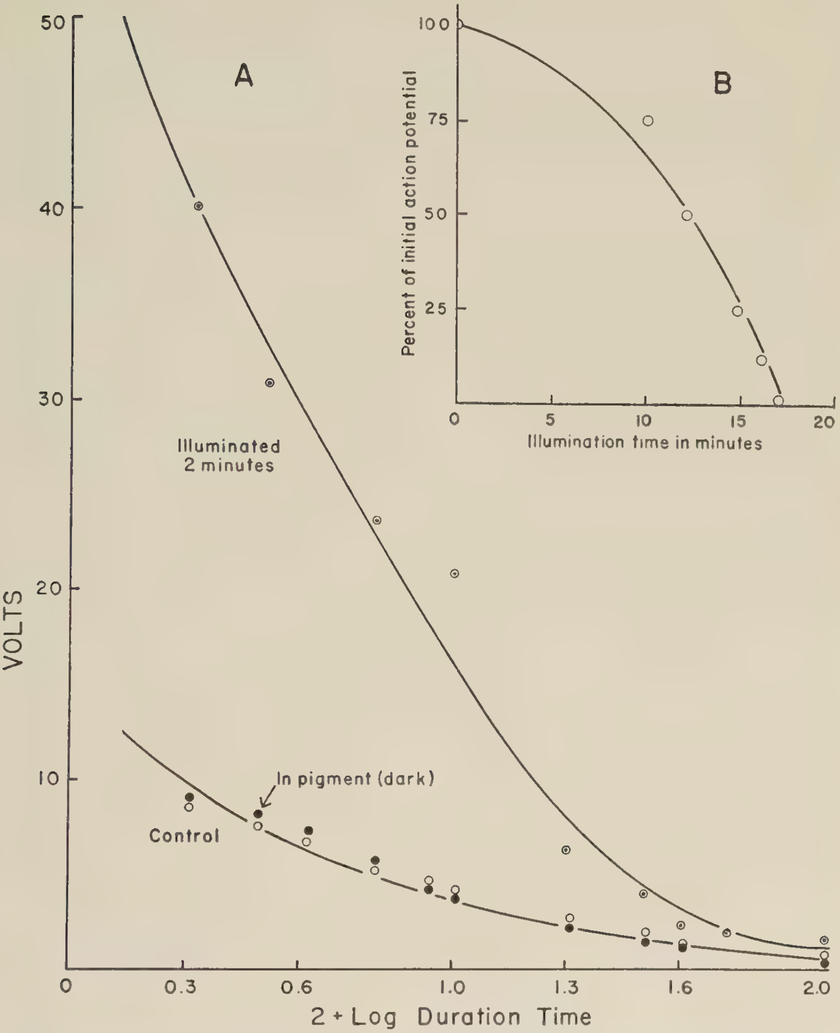

Fig. 1 A Strength duration curve for crab neuron bundle in Blepharisma pigment in the dark and in light compared to control before exposure of the bundle. The action potential disappeared after 4.5–5 minutes of illumination. The voltages recorded are not those reaching the fibers since much shorting occurs in the perfusion chamber.

B Decline of action potential following illumination of a small bundle of crab neurons in crab Ringer's solution saturated with Blepharisma pigment.

duration curves. Crab neurons are also not affected appreciably by illumination alone even for several hours.

However, in the presence of Blepharisma pigment illumination resulted in the decline of the action potential of crab nerve bundles. A slow decline is illustrated in figure 1 B; sometimes the decline is much more rapid. In groups of 4 to 12 fibers, the spike disappears in from 1 to 17 minutes, in the larger bundles slowly at first, then precipitously.

It was difficult to determine strength duration curves for single illuminated neurons, because the damage to single or small groups of fibers was so rapid. When measurements were made the threshold was found to rise soon after illumination, the rise continuing until the fiber stopped firing. Some data are given in figure 1 A for a group of fibers which showed no effect of the pigment in the dark but which ceased firing in just about 5 minutes of illumination.

To determine whether the Blepharisma pigment affected spike duration the time for a complete spike on a crab neuron was determined before and during illumination. For example, if the spike time was 7.5 to 8.0 milliseconds in duration before exposure, on illumination in the presence of Blepharisma pigment, it remained constant at 8.0 for 7½ minutes at which time the action potential fell to zero.

Photodynamic action of Blepharisma pigment also appears to have no effect upon the refractory period. For example, the refractory period before a spike equal to the preceding one could be elicited was 16 milliseconds before exposure. On illumination, in presence of Blepharisma pigment, it was 15.5 after 15 seconds, 15 after 2.5 minutes, and 16 after 3 and 4 minutes. The action potential declined to zero after 4½ minutes illumination. To produce a spike 50% of the original, the refractory period was 8 milliseconds before exposure and 7.8, 6.8, 7.8 and 7.7 for 0.25, 2.5, 3 and 4 minutes of illumination, respectively. The absolute refractory period was also similar before and after illumination (crab fibers).

In summary, it appears that the highly concentrated aqueous extract of Blepharisma pigment is harmless to bundles of crab or frog neurons in the dark but sensitizes them to light and causes injury and death by photodynamic action upon exposure to intense visible light. During illumination, injury to the neurons is manifested by decreased sensitivity and falling action potentials. However, spike duration and refractory period appear to be unaffected. The pigment does not visibly color the neurons either in the dark or in the light, although protozoans killed by it are colored (Giese, '49, '53).

2. *Effects of rose bengale.* Although the action potential of a sheathed frog sciatic nerve is unaffected in the dark by immersion in 1:1000 rose bengale for an hour, that of an unsheathed nerve fell to zero in that time, and the neurons appeared to be stained by the dye. The action potentials of small bundles of neurons fell to zero in half an hour in the dark, even in 1:10,000 rose bengale; the decline was much more rapid in 1:1000 rose bengale. Clearly, rose bengale is much more toxic to nerve in the dark than Blepharisma pigment. Not only did the action potential fall but the sensitivity of the nerve in the dark declined rapidly as shown by the strength duration curves. For example, after immersion in rose bengale 1:10,000 in the dark for 5 minutes, the threshold voltage required to stimulate the nerve was just about doubled all along the line. After that time the change of the short duration component was slower but the threshold continued to rise. At the long duration end of the curve little change occurred until the nerve ceased to function. In higher dilutions (1:100,000 and 1:1,000,000) injury was not observed for several hours.

When a frog preparation in 1:10,000 rose bengale was illuminated, the action potential fell to zero in 31 seconds; for a similar preparation in 1:100,000 it took 68 seconds; and in 1:1,000,000 it took two minutes and 16 seconds. Soon after illumination the threshold rose rapidly, indicating a loss in sensitivity of the neurons.

Results with bundles of crab neurons are similar to those for frog preparations, but since single fiber preparations were not employed in either case, quantitative comparisons cannot be made and details are therefore omitted. However, the results indicated that the photodynamic effect is alike in kind in the two types of fibers.

DISCUSSION

Photodynamic dyes are known to become adsorbed to surfaces from which they are often removed only with difficulty (Blum, '41). It is therefore not surprising that they should alter the action potential of the neuron even in the dark, since the action potential of the neuron requires the integrity of the surface of the fiber (Brink, '51). The time before the dye affects the nerve may be the period during which it is penetrating to the site of action. The more rapid action of the photodynamic dye, rose bengale, than of Blepharisma pigment upon the action potential of frog and crab neurons in light than in the dark suggests either that the dye penetrates more rapidly in the light, or that the photooxidation disrupts the surface of the cell.

The photodynamic dyes tested here in some respects act like certain narcotics which abolish the potential and decrease the sensitivity (Brink, '51). However, the refractory period and the spike duration are altered by narcotics but are not noticeably affected by the dyes either in the dark or in the light up to the time when the action potential fell to zero.

The relatively slower action of extracted Blepharisma pigment than of rose bengale on neurons of both crab and frog perhaps results partly from the lower solubility of the extracted pigment in Ringer's solution and partly because of its lesser uptake by the cells, since neither crab nor frog fibers stain noticeably in it. In contrast the fibers of both frog and crab stain deeply and rapidly in rose bengale, becoming intense red.

It is probable that photodynamic pigment enters the frog neuron at the nodes where the cell is essentially naked. Since rose bengale is more soluble in water than in lipoidal agents, its nodal entry into neurons seems more likely than passage through the myelin sheath. Nodal entry of a variety of vital dyes has been reported (Lehmann, '55). Since the node is important in functioning of myelinated neurons (Tasaki, '53), injury at the nodes changes the properties of the entire neuron.

The failure to obtain photodynamic effects on nerve illuminated in presence of Blepharisma pigment previously reported (Giese, '53), is interpretable on the basis of the present experiments. In an entire nerve both the sheath and the outer layers of neurons protect the neurons within the nerve bundle. It is only when small groups of fibers or single fibers are illuminated in the presence of Blepharisma pigment that photodynamic action becomes apparent.

The fact that the neurons tested are not susceptible to the Blepharisma pigment in the dark indicates their greater resistance to it than that of protozoans which are readily killed by equal concentrations of the pigment in the dark. The observation that even in bright illumination high concentrations of the pigment are necessary to injure the neurons tested indicates that both of these unmyelinated and myelinated neurons are much more resistant to the photodynamic effect of the pigment as well. The reasons for these differences have not been ascertained.

SUMMARY

1. A study was made of the effects upon action potentials, threshold and some other properties of the nerve of the bullfrog and of the crab, *Cancer productus,* of the photodynamic dye extracted from the ciliate protozoan *Blepharisma undulans,* and of the photodynamic dye, rose bengale.

2. Sheathed nerves of the frog are highly resistant to Blepharisma pigment, both in the dark and in the light. Small bundles of unsheathed nerves, while resistant to Blepharisma pigment in the dark, are rapidly injured when illuminated.

3. Nerves of the crab are not injured by Blepharisma pigment in the dark but are damaged in the light. The spike declines and the threshold curve rises but the refractory period and spike duration appear to remain about the same.

4. Sheathed nerves of the frog are highly resistant to the photodynamic dye, rose bengale, even in a concentration of 1:1000, both in the dark and light. Small bundles of neurons from unsheathed nerves are affected by long exposures to dye in the dark and are rapidly injured in the light even in dilute solutions of the dye, the rate of injury depending upon the concentration of the dye. The spike falls and the strength duration curve is altered, with a rise in threshold. The refractory period and spike duration appear to be little altered.

5. Small bundles of the crab nerve in the dark are highly sensitive to rose bengale at high concentrations in the dark. The strength duration curve indicates a rise in threshold. In light, lower concentrations of the dye quickly injure the neurons as shown by a decline in action potential.

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PHOTOREVERSAL OF ULTRAVIOLET INJURY TO FROG AND CRAB NERVES [1]

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FIVE FIGURES

Ultraviolet (UV) injury as manifested in suppression of cell division, or induction of mutations, is considered to be largely a nuclear phenomenon presumably resulting from suppression of desoxyribonucleic acid (DNA) synthesis which is limited to the nucleus. Photoreactivation appears to be the reversal of the suppression of DNA synthesis (Kelner, '53). Conversely, the effect of UV on ciliary movement which is a strictly cytoplasmic phenomenon appears to be very little if at all subject to photoreversal (Brandt and Giese, '56). Consequently it might be predicted that injurious effects of UV upon the nerve fiber lacking a nucleus should show no photoreversal. The experiments reported below indicate that UV injury to nerves is, in fact, photoreversed.

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[1] Supported in part by Contract AP(11-1234) with the Atomic Energy Commission; in part, during the terminal stage, by Public Health Contract C-1799(C2). We are indebted to Dr. F. Crescitelli of the University of California at Los Angeles for suggesting methods on stripping nerves, to Dr. T. Bullock of the same university for suggestions on the use of a perfusion chamber in which the nerve was irradiated and for initially suggesting this line of experimentation, to Dr. C. L. Prosser of the University of Illinois for suggestions of ionic tests, and to Dr. L. R. Blinks for making available facilities for this work at the Hopkins Marine Station during the summer of 1954. Preliminary reports on this work have appeared in abstracts (Pierce et al., '53, Anat. Rec., *117:* 643; Pierce and Giese, '54, Anat. Rec., *120:* 793).

MATERIALS AND METHODS

A large frog nerve is highly resistant to UV (Audiat, '31, '34; Boyarsky, '52) and in the present study the action potential of a frog sciatic nerve was unaffected by a 4 hour exposure to intense UV. Unsheathing it increased its sensitivity and the action potential fell by 20% in 8 minutes, but even exposure to UV for an hour beyond this had no further effect. Yet minute sheathed nerves from the throat of a frog were readily affected by UV, the action potential failing to zero within a few minutes. Presumably in a large nerve the thick sheath filters out the injurious UV, and, in an unsheathed large nerve, the outer fibers screen out the injurious UV, being themselves damaged but in turn protecting the inner fibers. It was therefore necessary to use bundles of a few fibers or single fibers in these studies.

The sciatic nerve in the thigh quickly dissected from a pithed bull frog, *Rana catesbiana,* was placed in Ringer's solution containing glucose (Sollman, '45). Under a binocular dissecting microscope the nerve was again cut with a sharp pair of scissors and the nerve cable protruding from the sheath (epineurium) was grasped with one pair of watchmaker's forceps and the sheath with another pair. The sheath was then gently stroked back away from the end and grasped firmly and stripped from the nerve bundle. Since a nerve so unsheathed gives the same electrical responses as before the operation, the method of stripping is considered satisfactory. According to Shanes ('54) the unsheathed nerve, except for being less stable, is comparable to the sheathed nerve and Crescitelli ('51) has even re-sheathed a nerve and shown its properties to be unchanged by the process.

The nerve cable was separated into smaller units from which, under a higher power, groups of neurons were removed. When a single fiber was desired, all the units in the group but one were removed or crushed, and the thin bundle was supported by white silk thread. Under such conditions discharges continued without change for 4 hours or more — a period longer than that of any of the radiation experiments.

Freshly caught commercial edible crabs *(Cancer magister)* were obtained daily for experiments. The nerve from the second podomere of the endopodite of the walking leg was dissected out by cutting the exoskeleton lengthwise on each narrow edge, snipping across the tendons, separating the muscles and lifting out the nerve, which was placed in crab Ringer's solution (Smith, '47). It was then separated into single fibers of which only giant fibers supported by a wet white silk fiber were used in experiments. Under such conditions the neurons continue to discharge without change for 4 hours or more, but are readily affected by UV as already reported by Gasteiger ('53). The crab fiber makes a better experimental preparation than the frog fiber because screening of UV by extraneous injured fibers is avoided.

To avoid fatigue artifacts, nerves were never stimulated more frequently than once a second, except in determination of the refractory period. When this was attempted, the stimulation was much more frequent but the nerve was subjected to such stimuli for only a relatively brief period of time.

The exposure chamber, consisting of a shielded plastic box containing the two stimulating electrodes, two pick-up platinum electrodes and a ground wire, was filled to within 1 mm of the electrodes with the appropriate solution. The covering quartz plate formed the top of a moist chamber within which the nerves continued to discharge normally in the dark for 6 hours.

An oscilloscope comparable to a Tektronix Type 514 synchroscope of the trigger-sweep type, a Tektronix Type 122 low-level Pre-amplifier and a Model S-4-B Grass stimulator were used. The intensity of radiations from a G-8-T-5 Westinghouse Sterilamp, which emits mainly the short UV wavelength 254 mμ, measured by a Hanovia UV meter (Model 603), was approximately 230 ergs/mm^2/sec. at the distance used. The entire nerve fiber was irradiated. The intensity of the photoreversing light source, a GE 100 watt mercury flood lamp, the light of which was filtered through 4 inches of copper chloride solution and Corning filter No. 4080 to eliminate the long ultraviolet, was 2200 footcandles at the surface of the chamber. The entire nerve fiber was illuminated. Illumination of crab nerve fiber preparations for as long as 4 hours caused at most a decline of about 5% in action potential. In the frog the decline was from 8 to 12%. Experiments on the frog nerve fibers were performed in a constant temperature room at 20°C. at Stanford, while those on the crab nerve were carried out at the prevailing room temperature of the Hopkins Marine Station (about 20°C.). Many repetitions were made for each dosage, the number depending upon the consistency of the results but usually up to 10 repeats were made. When inconsistent behavior was observed it is noted in the appropriate section.

RESULTS

1. Changes in action potential of frog nerve bundles following UV and light

The most obvious effect of UV upon the stimulated nerve fiber is the decline in the amplitude of the action spike as compared to a control in the dark which continues to discharge without change for 6 hours. Exposure of the nerve to UV is usually accompanied by an almost linear decline in spike amplitude (fig. 1). When irradiation ceases, the spike remains constant for a period which varies inversely with the dose, after which it declines almost to zero in three hours (fig. 2 C). A preparation kept in the dark after irradiation shows no increase in spike height except after very small dosages. When a nerve fiber is illuminated with visible light immediately after UV, the subsequent course of events depends upon the UV dosage to which it has been subjected. When the spike height of an irradiated nerve fiber has declined by 50% or more, illumination does not prevent decline to zero at about the same rate as in the irradiated dark control. When the decline in spike height of an irradiated nerve fiber is less than 50%, decided evidence of photoreversal is indicated either by a slower decline in spike or by increase in amplitude after illumination, especially when the decline was between 30 to 50% (fig. 2 A, B).

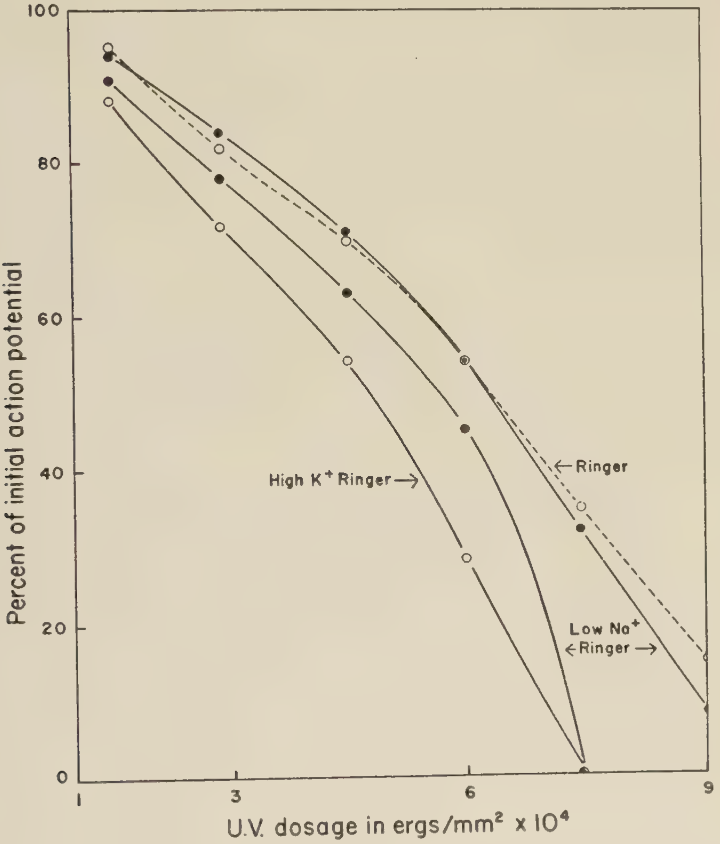

Fig. 1 Curves for typical experiments on the effect of ultraviolet radiations (UV) upon the action potentials of single frog nerve fibers (in a small bundle containing a few crushed fibers) in various salt solutions. In low sodium solution the effect of UV on the action potential is sometimes like that in Ringer's solution; more frequently injury is exaggerated. In high potassium solutions the decline in action potential after UV was always more rapid.

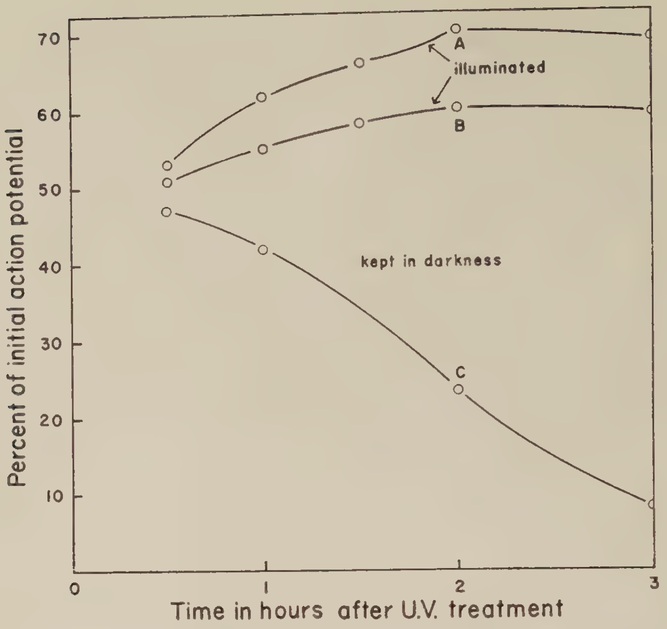

Fig. 2 Curves for typical experiments showing photoreversal following illumination of UV-treated frog nerve fibers. Index of UV injury was decline of action potential.

2. *Effect of ultraviolet and visible light on the action spike in crab nerve*

The spike potential of crab giant fibers irradiated with UV falls almost linearly with the dosage (fig. 3) in much the same way as that of frog nerve fibers except that smaller dosages are needed to produce an effect. Fifty per cent reduction of spike potential in 36 crab nerve fibers occurred after a UV dosage of 4.5×10^4 ergs/mm^2. This difference in susceptibility cannot be considered significant because the frog preparations contained a single intact fiber and several crushed ones which might protect the intact one from UV. The crab preparations were single fibers free of extraneous materials.

On cessation of irradiation the action spike remains constant for a brief period of time; then it falls slowly to zero

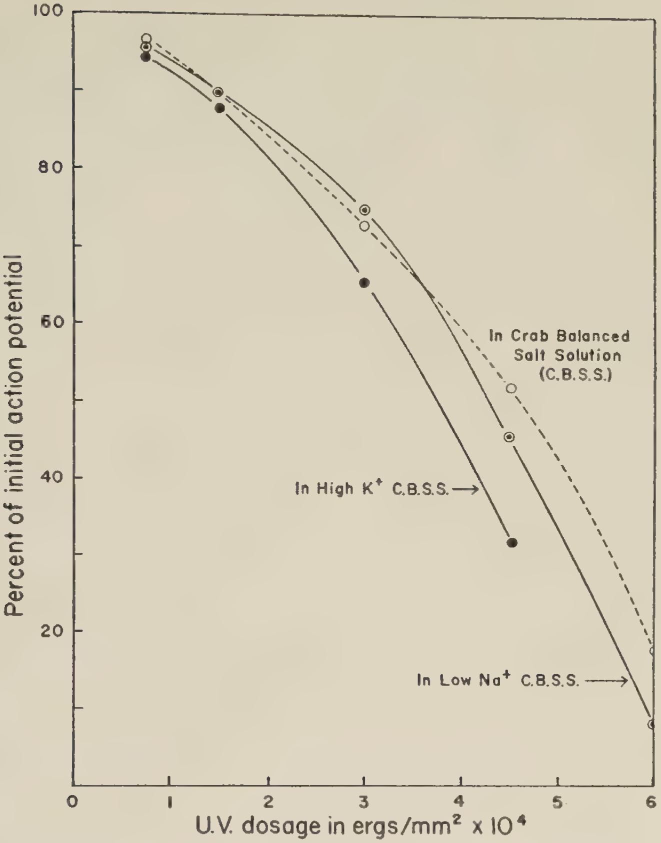

Fig. 3 Curves for typical experiments on the effect of UV upon the action potentials of single crab giant nerve fibers in various salt solutions. These preparations were free of injured fibers. The decline in action potentials is quicker in low sodium and high potassium salt solutions.

over a three hour period (fig. 4). In two exceptional fibers of 12 tested, the decline in action potential was less marked and after irradiation declined very slowly, remaining practically constant over the three hour period of observation after cessation of irradiation. No reason could be assigned to explain this behavior.

If the UV treated crab nerve fibers are illuminated as soon as the action potential has fallen by 50% or less, the action potential either remains constant or increases in magnitude, rising up to about 62% of the original size during the two hour period of illumination, after which it again declines slowly (fig. 4). The increase in magnitude of the spike after illumination is greatest for preparations in which UV has induced only a 40% decline in spike height. The UV-treated preparations in the dark decline sooner and more rapidly.

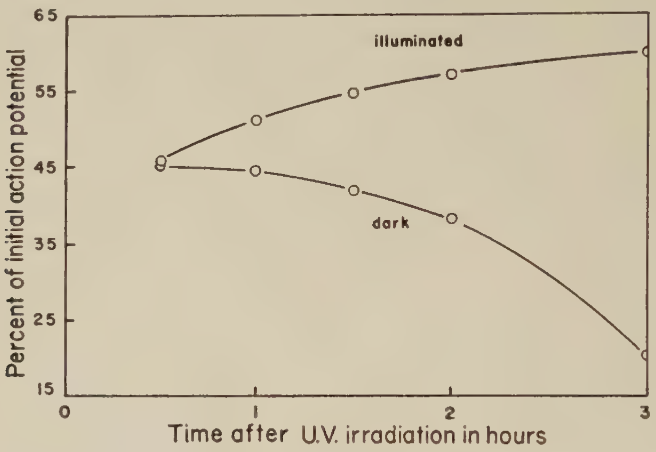

Fig. 4 Curves for experiments showing photoreversal following illumination of UV-treated single crab nerve fibers (average of 12 experiments). Index of UV injury was decline of action potential.

3. *Effects of ultraviolet and visible light on the strength-duration curve in frog and crab nerve*

To gain further insight into the nature of UV action upon the nerve fiber and its photoreversal, the strength-duration curve was determined first before, during UV action, and finally during illumination subsequent to irradiation, because the strength-duration curve is a measure of the sensitivity of the nerve to electric stimulation.

After exposure to a UV dosage of 1.5×10^4 ergs/mm^2, the short duration component of the strength-duration curve for a crab nerve fiber rises while the threshold in the long duration region falls (fig. 5). After illumination both components of the strength-duration curve return toward normal. After a larger UV dosage (6 to 8 × 10^4 ergs/mm^2) both portions of the strength-duration curve rise, indicating a loss in sensitivity on the part of the nerve (fig. 5).

The above evidence indicates that small UV dosages unstabilize the frog nerve fiber, making it more or less sensitive than the control depending upon the range or strength of the stimulus. After large UV dosages the nerve fiber becomes less sensitive. Ultraviolet radiation injury to nerve fibers

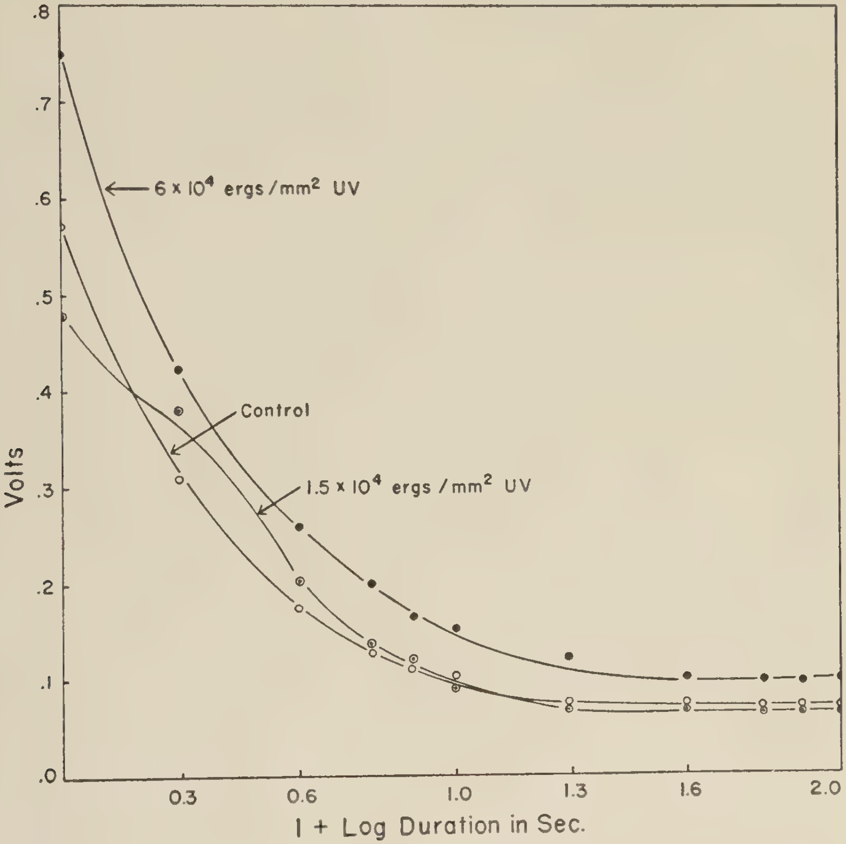

Fig. 5 One of 8 similar experiments showing changes in strength duration curves for a single crab nerve fiber following exposure to different dosages of UV. Data for 4.5 × 10^4 ergs/mm^2 are omitted for clarity, but the points fall close to the control especially for the short duration times. Photoreversal data are also omitted because the points lie close to the control points.

is reversed by illumination only if the dosage of UV has not already increased the long duration component of the strength-duration curve. Once this component has increased, illumination has no photoreversing effect.

Essentially similar results were obtained in a series of tests on frog nerve fibers as on the crab fibers, difference existing only in magnitude of dosages required. Since the details are of small moment they will not be recorded here.

4. *Effects of changed ionic environment on responses of nerve fibers to ultraviolet and visible light*

Ringer's solution rich in potassium (table 1) reduces the potassium gradient and the potential between the inside and outside of a nerve cell. One might therefore predict that on

TABLE 1

Ringer's solutions

Frog (weight in grams per liter)

CHEMICAL	NORMAL	HIGH K^+	LOW Na^+
NaCl	6.5	6.15	. . .
Choline	. . .	. . .	16.9
KCl	0.15	0.45	0.15
$CaCl_2$	0.15	0.15	0.15
$NaHCO_3$	0.20	0.20	0.20
$MgCl_2$	0.10	0.10	0.10
Dextrose	0.10	0.10	0.10
Water	to 1000 ml	to 1000 ml	to 1000 ml

Crab (weight in grams per 1100 ml)

CHEMICAL	NORMAL	HIGH K^+	LOW Na^+
NaCl	29.6	27.72	. . . .
Choline	. . . .	. . . .	74.38
KCl	0.99	2.92	0.90
$CaCl_2$	1.48	1.48	1.48
$MgCl_2$	0.42	0.42	0.42
$MgSO_4$	2.02	2.02	2.02
H_3BO_4 0.5 M	20 ml	20 ml	20 ml
NaOH 0.5 M	0.96 ml	0.96 ml	0.96 ml
Water	to 1100 ml	to 1100 ml	to 1100 ml

stimulation the tendency for the neuron to lose potassium from the inside of the cell would be decreased in such a solution. Therefore, the potential of UV irradiated nerve fibers should fall less readily in it. However, the converse was found in all 4 experiments tried with frog nerve fibers (examples in figs. 1 and 3). Furthermore, illumination of the nerve fibers treated with UV in potassium-rich solutions did not in any case arrest or reverse the UV injury even if they were washed in normal Ringer solution before illumination. The strength duration curves of frog nerve fibers irradiated in high potassium Ringer solution resembled those for fibers irradiated in normal Ringer, except that in all cases the effects of irradiation appeared at lower dosages.

Although it might be predicted that placing a nerve fiber in a Ringer solution low in sodium (table 1) should reduce the action potential because upon stimulation the entry of sodium into neurons contributes to the action potential (Hodgkin, Huxley and Katz, '49), the action potential was in fact little affected. On the other hand, the sensitivity to UV of the nerve fiber immersed in low sodium Ringer usually appeared to be increased (figs. 1 and 3). In 7 of 12 preparations in low sodium Ringer, the action potential was affected to the same degree at first as in fibers kept in normal Ringer, but soon became erratic and the fiber ceased firing when the action potential fell to 60% of the initial value. In later stages of irradiation only two of 12 in low sodium Ringer were as resistant as those in normal Ringer. Photoreversal of UV injury of fibers treated in low sodium Ringer was obtained in no case, even after washing in normal Ringer before illumination.

Strength duration curves for frog nerve fibers in low sodium Ringer rose almost immediately after even relatively small doses of UV (1×10^4 ergs/mm^2), indicating a decline in sensitivity of the neurons. The short duration component then became erratic, indicating a general breakdown of the conducting mechanism of the neurons.

Crab neurons in high potassium and low sodium behaved much as the frog preparations when subjected to UV. While the results were equally consistent, they were less marked and are therefore not documented here.

5. Effects of ultraviolet radiation on refractory period and spike duration

In 7 preparations of frog nerve, the time for complete abolishment of the second spike (absolute refractory period), the time for 50% abolishment of the second spike, and the delay time at which the second spike was equal to the first were determined before and after exposure to UV of various dosages. Within the limits of error (12, 18 and 15% respectively) no systematic changes were observed over the entire period of observation until the nerve fiber ceased firing. Similar results were obtained with crab neurons.

In 16 preparations of frog nerves studied before and after treatment with UV, no detectable change in spike duration could be observed from photographic records up to the time when the spike disappears. Since the method is good to about 5%, the conclusion may be drawn that spike duration is little if at all affected by exposure to UV. Similar results were obtained with crab neurons.

DISCUSSION

The evidence presented in this paper for photoreversal of decreased action potential and sensitivity of ultraviolet treated neurons of crab and frog indicates that photoreversal is not restricted to the nucleus or nuclear area since the fibers used were severed from the cell body containing the nucleus. Nor is photoreversal restricted to structures containing desoxyribonucleic acid (DNA) since DNA is either lacking in nerve fibers or present in very small amounts. However, ribonucleic acid (RNA) is present in large amounts and plays a role in nerve metabolism (Caspersson, '50; Gerard, '55). It is conceivable that RNA production or utilization is retarded by

UV and in turn is reversed by visible light. Other evidence of inhibition of RNA synthesis by UV and its photoreversal by visible light has been accumulated for microorganisms (Iverson, unpublished). However, the action spectrum for UV effects on nerve (Booth et al., '50) with maxima at 297, 280, and 265 mμ, the middle peak being the highest, does not point unequivocally to any one protoplasmic constituent as the locus of UV injury, although thiamine is suggested by the authors as one of these.

Since the amount of photoreversal of UV injury to nerve is less striking than that of cells which have been retarded in division or stopped by UV (Kelner, '53), the action of UV on nerves is either qualitatively different from that which is affected by UV in dividing cells or the process being affected plays a less important role in development of action potentials.

The data taken as a whole suggest that the neuron might be especially useful for analysis of one aspect of photoreversal, that is of the system outside the nucleus. In further studies it would be useful to determine the effect of UV and its photoreversal by visible light on the resting and action potentials between electrodes inside and outside the neuron to gain further insight into the nature of the process. Such experiments are planned. The results may also be of interest to investigation of transport of salt ions across the cell membrane, a study already in progress elsewhere (von Muralt, '53, '54; von Muralt and Stampfli, '53).

SUMMARY

1. Control nerve fibers on electrodes in the chamber used, when stimulated, discharge without decline for at least 4 hours. When illuminated with visible light of the same intensity as that used for photoreactivation from ultraviolet (UV) injury, the action spike of frog nerve fibers declined approximately 8 to 12% in 4 hours; the potential of crab fibers fell only 5% in 4 hours.

2. The action spike (1 per second) of both frog and crab fibers exposed to UV declines almost linearly as irradiation

proceeds. After a small UV dose, spontaneous recovery may occur in the dark. After large UV doses the action spike remains constant for a time, then declines to zero after a few hours in the dark.

3. When a UV-treated nerve fiber, the spike of which had fallen by about 50% or less from the original, was illuminated with white light for two hours the UV injury was reversed to the extent that the spike returned about 15% toward the original height. After greater decline in spike height following UV, illumination proved of no avail.

4. When UV affects the short duration component of the strength-duration curve of the frog nerve, photoreversal is still possible, but when the long duration component is also affected, it is not. Somewhat similar results were obtained with crab nerve, but photoreversal was observed only when the action potential had not been reduced by UV more than 40%.

5. Within the limits of error no change in the refractory period of either frog or crab nerve fibers could be detected photographically after UV treatments.

6. No change in spike duration of either frog or crab nerve could be detected photographically after UV treatment although x-rays have a marked effect of this type (Gerstner et al., '55).

7. Nerve fibers in Ringer enriched with potassium are more sensitive to UV than those in normal Ringer. No photoreversal was observed in crab and frog nerve fibers kept in potassium-rich balanced salt solutions.

8. Frog and crab neurons treated with UV in a balanced salt solution low in sodium were generally more sensitive to UV than those in normal Ringer, at least after larger doses. No photoreversal was obtained in fibers kept in such solutions during irradiation.

9. The results are discussed with relation to data in the literature.

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SINGLE FIBER GUSTATORY IMPULSES IN RAT AND HAMSTER

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FOUR FIGURES

INTRODUCTION

Some properties of taste receptors have recently been analyzed by observing the integrated action potentials recorded from the chorda tympani nerve when the tongue is bathed by stimulating solutions (Beidler, '53; Beidler, Fishman and Hardiman, '55). The present research was undertaken to determine to what extent the integrated response reflects the single unit activity in the nerve and to investigate and evaluate the characteristics of single unit activity in response to various stimuli.

METHOD

Records were obtained from the chorda tympani of hamsters, of male and female Wistar and Sherman albino rats and Lashley hooded rats. Animals were anesthetized with sodium nembutal (20 mg per kilogram body weight). The nerve was exposed, freed from surrounding tissues and cut at the point where it enters the bulla (Beidler, '53). Small strands of nerve with one or two functional axons were dissected from the peripheral end of the free nerve and placed on cotton wick electrodes (Beidler et al., '55). The anterior two-thirds of the tongue, which contains the fungiform papillae innervated by afferent axons of the chorda tympani, was placed in a small glass flow-chamber through which a uniform flow of test solution could be passed, bathing a constant area of the tongue. Test solutions were flowed over the tongue for 15 to 20 seconds at the rate of 3 to 5 ml per second. One to two minute tap water rinses were used between successive stimulations. The impulses were amplified and displayed on an oscilloscope and photographs with moving film were taken with a Grass kymograph camera. The following test solutions, in concentrations from 1.0 molar to 0.0005 molar, were diluted from stocks of 1.0 molar concentration: NaCl, LiCl, $MgCl_2$, $CaCl_2$, KCl, NH_4Cl, $SrCl_2$, $BaCl_2$, NaBr, $NaNO_3$, Na_2SO_4, and Na_2CO_3. Stock solutions were titrated or analyzed gravimetrically to assure accuracy within 0.5%. Five-tenths M NaCl, 0.01 N HCl, 20 mM quinine hydrochloride and 1.0 M sucrose served as stimuli for the 4 taste qualities, salt, sour, bitter and sweet. Soluble saccharin was also used on preparations which exhibited reasonably good response to sugar. Stimulation with each solution was repeated several times during an experiment, beginning with the lower concentrations. Controls of 0.5 and/or 0.1 M NaCl were used after every 4th or 5th stimulation. The data represent an analysis of almost 1000 separate stimulations.

Many investigators have used the response of a fiber during the first second of stimulation as their basis for comparison and analysis. This author has found that in the case of gustation, the initial response of a given fiber to a given stimulus may be somewhat variable, although the magnitude of steady-state activity is reproducible from one stimulation to another. Also, different fibers which have the same steady-state of activity after the first second or two of stimulation with a given stimulant may exhibit quite dissimilar initial activities. For this reason, response-concentration curves and other analyses for this paper were all derived using the average number of impulses over a longer period of stimulation (10 seconds or more) in lieu of initial response activity. The longer period is also convenient in comparing single fiber data with integrator data obtained from whole nerve preparations. The term "Receptor Unit" as used in this paper is defined as the receptor end-organ or organs innervated by a single chorda tympani fiber.

RESULTS

Nature of the response. Figure 1 illustrates graphically some of the variations in response of single units encountered in this investigation. Types of response obtained may be summarized as follows:

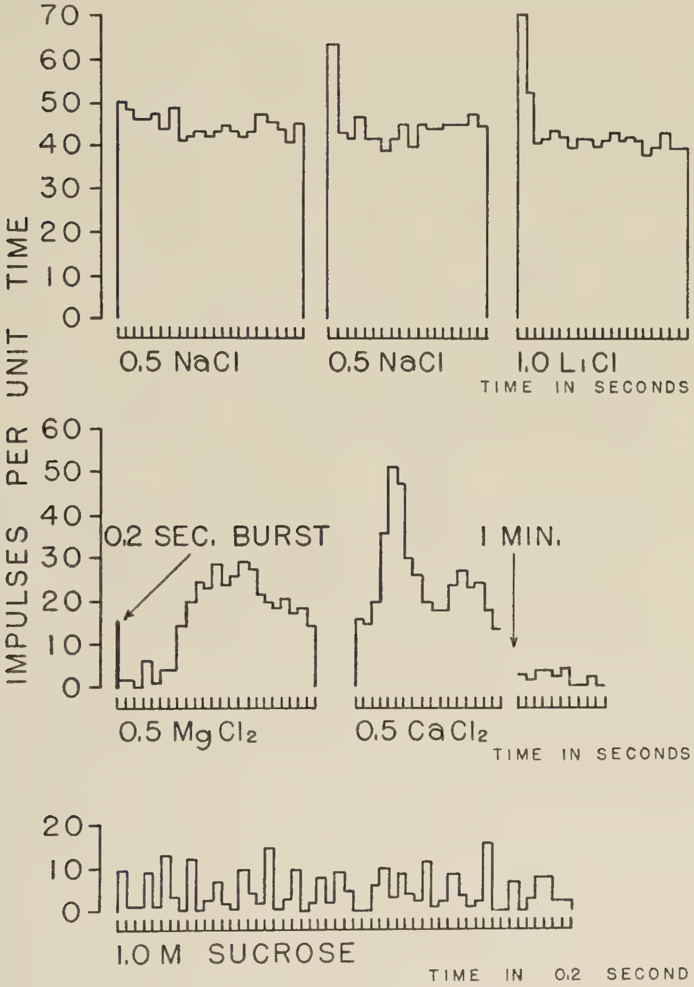

Fig. 1 Graphs illustrating typical single-fiber responses from chorda tympani nerves of rat and the temporal variations in each response. The two NaCl responses are from the same single fiber. All other responses are from different fibers. The horizontal plateaus in each histogram represent by their height the number of impulses during the corresponding unit of time along the abscissa.

1. Rapid initial response of one to two seconds duration followed by a rapid decrement to a steady state of activity which shows little evidence of fatigue on constant stimulation for three minutes or longer.
2. Steady state activity as in 1 but attained in the first second without rapid initial burst.
3. Rapid initial burst of 1/10 to 1/5 second duration followed by a short period of silence then a prolonged increase in activity to a maximum with a subsequent slow decline to some low-level steady state or to zero activity.
4. Gradual increase of activity from time of stimulation to a maximum followed by decline as in 3 above.
5. Rhythmic burst-like firing in which the impulses are grouped together with a fairly constant interval between groups.

Monovalent salts always evoke responses of type 1 or type 2. Some monovalent salts, for example LiCl, always elicited type 1 response in all fibers for which they were adequate stimuli. Others, for example NaCl, may elicit both types 1 and 2 response at different times in the same fiber and in different fibers. Divalent salts, in general, gave the type 4 response. $MgCl_2$ and $CaCl_2$ elicited type 3 response in some fibers and type 4 response in others. Type 5 response was obtained in a few fibers which responded particularly well to sweet substances. Beidler ('53) has also observed type 5 response. In the rat, fibers which responded in this manner to sweets, also showed the same type of response to some salts. However, the bursts were not so clearly delineated for agents which evoke a smaller frequency of response than sugar in these fibers. When the concentration of the sweet stimulant is increased in such a fiber, the number of impulses per burst increases and the time interval between bursts decreases. Neither factor is alone responsible for the overall increase in activity.

For the fibers for which sucrose was not an adequate stimulus, saccharin behaved as though it were a salt; its response-concentration curve was similar in shape, but lower than, the NaCl curve. For the fibers for which sucrose elicited an appreciably greater response than NaCl or NH_4Cl, saccharin behaved as though it were a sweet agent; its response-concentration curve paralleled that of sucrose and in one instance was slightly higher than sucrose. (It is possible that saccharin evokes a sweet taste when stimulating receptors especially sensitive to sucrose and a salt taste when stimulating receptors sensitive to salt but not to sucrose.)

Spontaneous activity of any appreciable amount was not noted in fibers of the rat and hamster. Only occasionally, after long stimulation with high concentrations of salts or acids, a low level of activity resulted, requiring several minutes of careful rinsing to eliminate completely.

One phenomenon encountered in all fibers, and with all stimuli, concerns temporal variations during the steady state of response (fig. 1). The response level of any given fiber at the steady state shows large, brief temporal variations during stimulation although the overall magnitude of response to a given stimulant is constant and reproducible in a fiber if averaged over several seconds.

Taste qualities. The substances that elicit the 4 taste sensations, salt, bitter, sour and sweet, are represented in these experiments by 0.5 M NaCl, 20 mM quinine hydrochloride, 0.01 N HCl and 1.0 M sucrose respectively. Thirteen fibers from the rat and 4 from the hamster were investigated with regard to the relative effectiveness of these 4 substances as stimulants for the receptor unit. Figure 2 presents a tabulation of the data obtained. The height of the column representing each substance indicates the average number of impulses per second for a period of 10 seconds stimulation.

Inorganic salts. A cation series of equimolar chloride salts can be used to reveal the effect of the cation of a salt on the response of the chemoreceptor. Likewise, a series of equimolar sodium salts can be used to reveal the effect of the anion. Kionka and Strätz ('22) concluded from behavioral experiments that the *intensity* of a salt depends on the cation and that the anion is responsible for the *character* of the salt.

Beidler ('53) recorded from a population of chemoreceptors and concluded that it is the cation which has the greater effect in stimulation of the receptor. In order to determine whether or not Beidler's population results could be applied directly to all single receptor units, similar experiments were performed with single nerve fibers. The effectiveness of all the anions investigated (Cl, Br, SO_4, NO_3 and CO_3) does not vary greatly (table 1) whereas the cations used (Na, Li, NH_4, Ca, Mg, K, Sr and Ba) show a very wide variation in effectiveness.

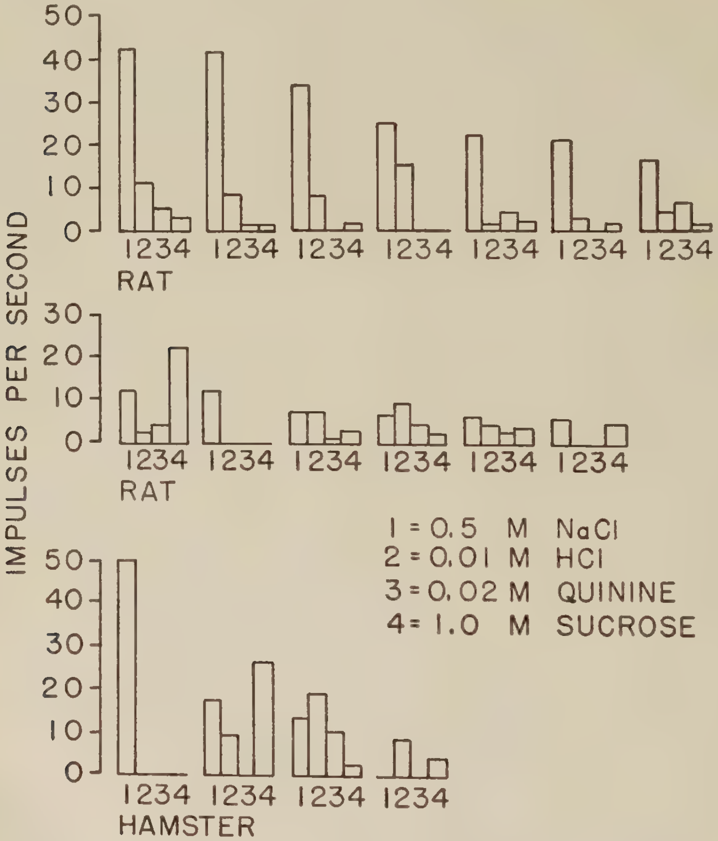

Fig. 2 Histograms showing the relative response of 13 single chorda tympani fibers of rat and 4 of hamster to stimulation of the tongue with substances representing the 4 taste modalities.

Figure 3 represents a tabulation of the data obtained from 7 fibers of rat and 3 of hamster for stimulation with inorganic chloride salts. The height of the column representing each substance indicates the average number of impulses per second for a period of 10 seconds of stimulation.

Temperature effects.[1] Several tests were performed to determine in what manner taste receptors respond to temperature. It was found that water alone at 5°C. evokes an initial response almost equal to that obtained with 0.1 M NaCl at 25°C. whereas tap water alone at 25°C. did not elicit a response in any of the fibers. Continuous stimulation with water at 5°C. results in a gradual decline in activity to zero or near zero. No activity was obtained with water alone in taste fibers of the rat and hamster from 17° to 49°C. One-tenth M and 0.5 M salts at 5°C. evoke a lower initial rate of activity than normally obtained with the given salt at 25°C. Integrator studies were also made with whole chorda tympani nerves of rat to determine the effect of temperature on the whole receptor field. Results obtained with water and NaCl at concentrations of 0.1 M and above at temperatures of 3° to 5°C. were similar to those described for single fibers. Goudriaan ('30) has shown that decreasing the temperature increases the intensity of salt taste for man. If the intensity

TABLE 1

Relative effectiveness of anions

0.5 M SALT	RESPONSE IN NUMBER OF IMPULSES/SECOND				
	Fiber	A	B	C	D
NaCl		8	23	7	28
NaBr		9	21	7	29
Na_2SO_4		6	20	6	23
$NaNO_3$		8	21	5	27
Na_2CO_3		5	20	6	28

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[1] Integrator studies on temperature were supported by grant No. G1732 from the National Science Foundation. More detailed studies on the effect of temperature are now in progress and will be reported in a later paper.

of sensation is proportional to the activity of the taste fibers then it would seem from these experiments that decreased temperature of salts (at concentrations of 0.1 M and above) would result in a decrease in the intensity of salt taste for rats and hamsters.

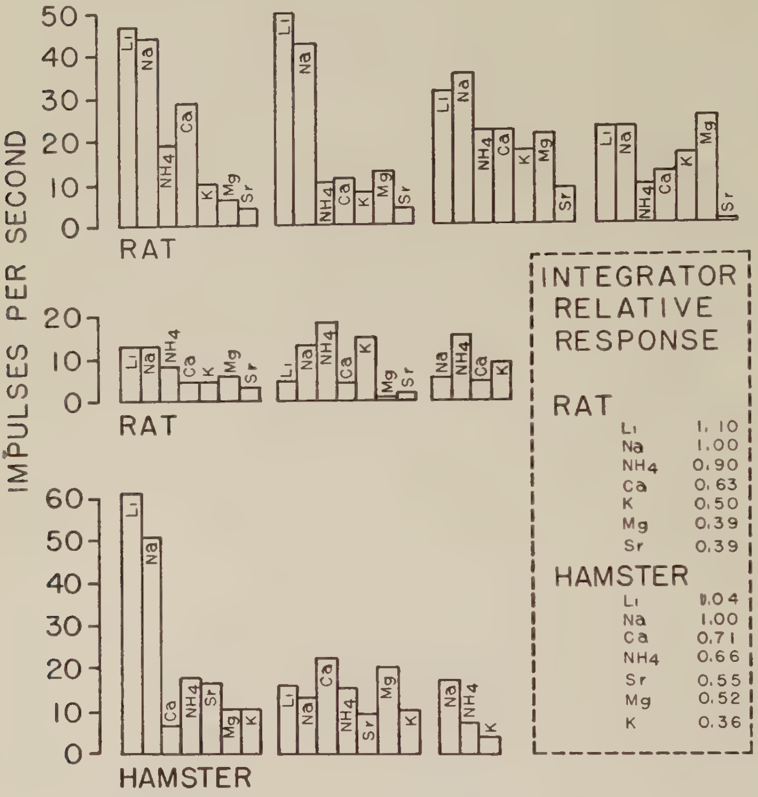

Fig. 3 Histograms showing the relative response of 7 single chorda tympani fibers of rat and three of hamster to stimulation of the tongue with 0.5 M Chloride salts.

DISCUSSION

Data obtained with an integrator show that sweet and bitter response in the rat are all low (less than 30% of the magnitude of 0.5 M salt response), with acid eliciting a slightly higher response than either quinine or sucrose for the concentrations used. This is not always the case with single fibers (fig. 2). Although the majority of the single fibers studied showed a salt response considerably greater than to stimuli associated with the other modalities, there were several fibers for which the salt response was low, one for which the acid response was relatively high and one for which the sucrose response was much greater than salt. In two fibers 20 mM quinine elicited a greater magnitude of response than did 0.01 N HCl, which is not the case with whole-nerve preparations. Even in the hamster, from which only 4 fibers were studied, one fiber responded extremely well to NaCl but not at all to HCl, quinine or sucrose. Still another did not respond at all to NaCl or quinine but fairly well to acid and sucrose. Hamster whole-nerve response to 0.01 N HCl is always a little more than 40% of the response to 0.5 M NaCl.

It is apparent that the fibers cannot be grouped according to modalities in any strict sense. A partial grouping can be made, however, if one allows for a certain overlap between groups. This overlapping in the distribution of the modalities from one fiber "group" to another has led some investigators to favor Frings' general theory of taste spectrum (Frings, '46, '48, '51) in lieu of the classical Four Modality theory. This view is taken frequently because the Four Modality theory is often based on the assumption that each primary modality depends on the existence of a specific type of end-organ, thus the existence of 4 primary types of end-organs. At this time we know of no morphological differences between taste buds which would account for specificity. This does not, however, preclude the existence of some functional differences. In the rat the majority of fungiform papillae contain but one taste bud (Fish, Malone and Richter, '44) consisting of several sensory cells. Foley ('45) counted 1157 sensory axons in one chorda tympani nerve of the cat, and it is known that some of these subserve senses other than taste. There are 575 buds on one side of the cat's tongue; therefore, there are at most two nerves per bud. However, each bud has at least 5 to 7 sense cells. If this argument holds for the rat as it does for the cat and dog, then one possible reason for the observed differences in single-fiber response is that there is probably more than one receptor cell innervated by each single taste fiber and the several individual receptors associated with a single fiber may respond differently to the stimuli associated with the 4 modalities. Therefore, the assumption of the existence of only 3 or 4 types of receptors may be valid in spite of the differences in single-fiber response.

Frings proposes that, ''The 'primary modalities' of taste might . . . be simply points of familiarity in an unbroken series of stimulative values.'' That is, modality depends on the stimulating power of the agent used. Thus, activity of only the most easily stimulated receptors, those of lowest threshold, would be interpreted as sweet; stimulation of these plus other receptors with higher thresholds would evoke a salty taste; these plus additional receptors would be interpreted as bitter, and finally, a substance which stimulates all or nearly all of the receptors would be interpreted as sour. The theory does not offer any obvious explanation for such fibers as those whose frequency of discharge to sucrose is considerably greater than to stimuli associated with any of the other modalities, and the effect of changes in concentration is mentioned only with regard to shifts in taste quality. The theory is based upon taste being related to only one parameter, rate of penetration or adsorption, and its continuous change with stimuli produces a complete spectrum. This is similar to vision in that color changes as there is a change in wavelength. ''Taste quality,'' as Frings states, ''would be determined actually from the *distributional* pattern of stimulation of quantitatively different receptors.'' Certainly there is little or no temporal pattern to most single-fiber responses in taste. It is the opinion of this author that the results of experiments presented in this paper and those of Pfaffmann and others do not agree with the Spectrum hypothesis. It would seem that a more tenable conclusion would be that the data point with favor to the Four Modality theory, with perhaps some minor modifications.

If the level of activity for a given period of stimulation is plotted against the molar concentration of the stimulant, a curve illustrating the relationship between nerve activity and concentration is obtained. Curves from rat single fibers with NaCl stimulation are almost identical in shape with curves obtained on whole-nerve preparations with the integrator (Beidler, '53). If the data are plotted as the ratio of concentration to response versus concentration, straight lines are obtained and the similarity of the curves is evident (fig. 4). Beidler ('54) has proposed a theory of taste for

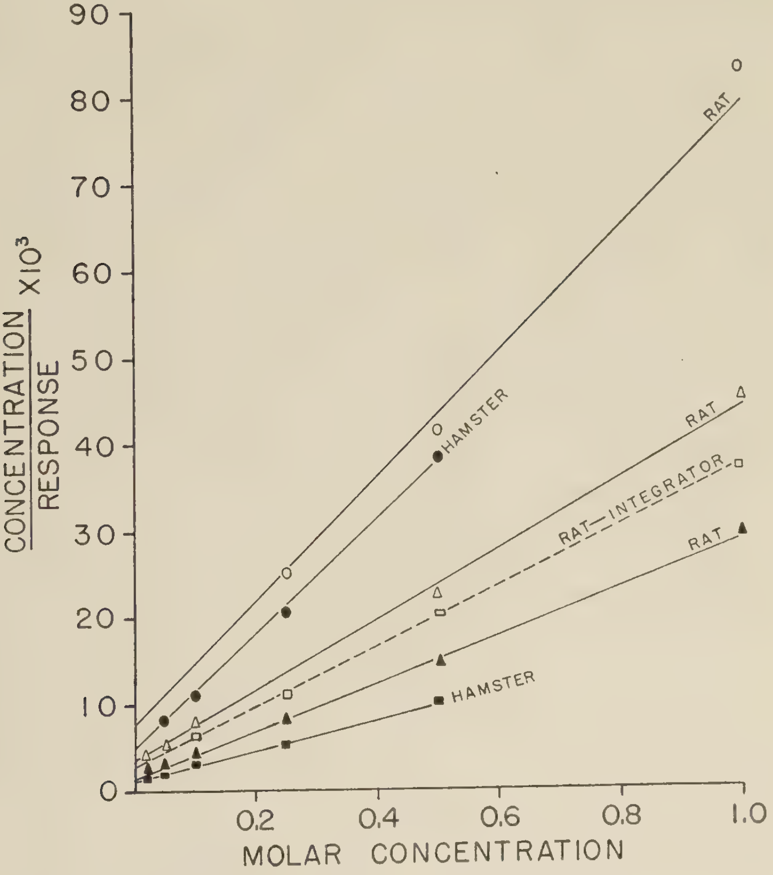

Fig. 4 NaCl concentration curves for rat and hamster. Each curve is for a different nerve fiber.

salts based on the assumption that the ionic species of salts fill given sites on some larger molecules (perhaps polyelectrolytes containing appropriate phosphate groups) of the taste organ. If it can be assumed that the response-concentration curve measures the association of the salt with the receptor surface of the cell, then an equilibrium constant for the association can be estimated. From an analysis of the concentration curves for various salts Beidler concludes that the mechanism is one of physical adsorption. An analysis of single-fiber curves obtained in the present investigation results in equilibrium constants ranging from 10.2 to 19.2 for NaCl and from 1.4 to 2.4 for KCl. These constants are the same order of magnitude as those reported by Beidler.

The binding of various salts is probably dependent upon the over-all configurations of the receptor molecules, the available side chains, and the proximity of neighboring molecules. That is, each receptor unit may have sites peculiar for the attraction of specific ions or molecular groups and the possibility of stimulation with specific agents depends upon the number of sites present in the particular unit for each agent. There is no reason to assume that, for example, a receptor must contain all sugar sites with no other sites present, or that a uniform mixture of sites will be found from one receptor to another. One would conclude that any combination or mixture may occur. Thus, each single fiber shows a mixture of modality sensitivities that is different from other fibers. A sweet sensation would result when the receptors most sensitive to sugars are stimulated, as in Frings' theory. An increase in concentration would result in an increase in frequency of discharge as more sites on the receptor were filled and also additional receptors with higher thresholds for sugar would be stimulated. The net result would be an increase in *intensity* of sweet taste but there would be no reason to assume a change in quality expressed either as an increase in discharge frequency of a given fiber or an increase in the number of fibers responding. In fact, no quality is discernible, as such, experimentally in a single fiber merely by the frequency of discharge. Quality discernment, such as sweet, for example, could only occur if those receptors with predominantly sweet sites end in a "sweet" area of the brain, etc.

The practice of using thresholds to express the "sensitivity" of receptors is a dangerous one. A receptor may respond to more than one modality and the relative thresholds may not accurately reflect the relative response at some higher concentration. There may, for example, be a receptor which responds very well to both sugar and salts but with sugar exciting at a much lower concentration. The saturation (maximum) level of response for salts, however, may be much higher than for sugar. In this case the threshold values would not be indicative of stimulation ability at all concentrations. We know of fibers which have a lower threshold to bitters, such as quinine, than to other substances. These responses to bitter substances always saturate at very low frequencies of discharge, and though the threshold to salts in these fibers may be much higher, the maximum response to salts is always at a much greater frequency than to bitter. Thus, the fiber may be classed as bitter if threshold values are considered but again may be classed as salty if saturation levels are considered. This shows the necessity of obtaining concentration curves for each substance used as a stimulant.

Although the individual single fibers are quite different in sensitivity they exhibit to each of the 4 primary taste qualities, the response of the whole chorda tympani nerve, as revealed by integrator studies, is characteristic and is constant and reproducible within a given species. None of the 4 hamster fibers examined, and few of the rat fibers, agree individually with the integrator data relative to all the taste qualities. However, the integrator expresses the *sum* of activities of the fibers making up the whole nerve bundle. If the activity of each of the single fibers in response to NaCl are added together (and similarly for each of the stimuli associated with the other three qualities) it is seen that the relative magnitude of response for each substance, as compared to NaCl taken as unity, is remarkably similar to the result of the integrator studies (table 2). Likewise, not all single fibers give identical cation groupings (fig. 3). There is considerable variation in the sensitivity to any given salt from one fiber to another. This implies that the sites of stimulation on the receptors are not always identical in physicochemical characteristics even where a single modality is considered, but that variations occur. These variations are graded; that is, are of different degrees. Thus, differences in molecular structure of the receptor surfaces (molecular configuration, available side chains, etc.) not only are present from one species to another (as shown by Beidler, Fishman

TABLE 2

Relative response to the modalities

HAMSTER INTEGRATOR DATA [1]		SUM OF ACTIVITY OF HAMSTER SINGLE FIBERS [1]	RAT INTEGRATOR DATA [1]	SUM OF ACTIVITY OF RAT SINGLE FIBERS [1]
0.5 M NaCl	1.00	1.00	1.00	1.00
0.01 N HCl	0.42	0.44	0.30	0.28
20 mM quinine	0.16	0.13	0.10	0.10
1.0 M sucrose	0.38	0.40	0.20	0.17

[1] Response is given as the ratio of test solution response to 0.5 M NaCl response.

TABLE 3

Relative response to salts

0.5 M SALT	SUM OF RAT SINGLE FIBER RESPONSES [1]	INTEGRATOR RESPONSES [1]
NaCl	1.00	1.00
LiCl	1.03	1.10
NH_4Cl	0.51	0.90
$CaCl_2$	0.45	0.63
KCl	0.40	0.50
$MgCl_2$	0.40	0.39
$SrCl_2$	0.10	0.39

[1] Responses given as ratio of test solution response to 0.5 M NaCl response.

and Hardiman, '55), but smaller variations also occur from receptor unit to receptor unit in a given species.

Although the fibers vary greatly one from another in their response to a series of salts, a sum of their responses, as with the modalities, approaches agreement with the integrator data (table 3). This would lead one to assume that as more single-fiber data are added to the ones studied here, the sums of their activities would approach even more closely the integrator data. The validity of this assumption is corroborated by the addition of data recently obtained by Pfaffmann ('55) on single-fibers from cat.

SUMMARY

The electrical activity of single fibers of the chorda tympani nerves of rat and hamster resulting from stimulation of the tongue were recorded and analyzed. Fiber activity indicated considerable variability in the sensitivity of the receptors both to substances representing the 4 primary modalities of taste and to various chloride salts. The relative position of a particular salt within a series varied with different receptor units. Activity to all stimuli showed brief temporal variations during stimulation.

Five general types of response, the responses characteristic of specific stimuli and the effect of low temperatures without chemical stimulation are described. By appropriate summation of overlapping single-unit responses, curves similar to the integrated whole-nerve responses have been produced. The data, as related to Frings' Taste Spectrum theory and the Four Modality theory, are discussed.

ACKNOWLEDGMENT

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THE EFFECT OF IODIDE AND THIOCYANATE IONS ON THE MECHANICAL AND ELECTRICAL PROPERTIES OF FROG MUSCLE[1]

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THREE FIGURES

The link between the action potential of the striated muscle fiber and the contractile process which follows within milliseconds is not known. Almost all stimuli which produce propagated or non-propagated contraction in excitable muscle have one common feature: the reduction of the membrane potential of the muscle fiber (Katz, '50), but no adequate explanation of how this surface depolarization causes a rapid change in the contractile proteins of the interior has been advanced. Each theory has been followed by detailed experimental refutations (Sandow, '52; Sten-Knudson, '54).

The depolarization in a propagated action potential has been interpreted as a sequential movement of ions across the membrane — i.e. a net influx of sodium ions followed by a net efflux of potassium ions, with only a small contribution of chloride ion to the net current (Hodgkin and Huxley, '52; Keynes, '51). The experiments have been more extensive on invertebrate nerve fibers than on striated muscle fibers, but the major features of conduction in many nerves and muscles seem so similar that it has been convenient to suppose that what is true for the squid giant nerve fiber is probably true for the frog sartorius muscle fiber. In the sartorius, as in the isolated squid fiber, the ''active membrane potential'' is linearly related to the logarithm of the sodium ion concentration in the surrounding fluid (Nastuk and Hodgkin, '50).

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Kahn and Sandow ('50) have described the marked increase in isometric twitch tension which results when the chloride ion of Ringer fluid is replaced by nitrate, bromide, iodide, or thiocyanate ion. Hill and Macpherson ('54) and Ritchie ('54) have confirmed and extended these observations. The rapid onset of twitch potentiation in bromide, nitrate, or iodide-Ringer, without appreciable change in the force-velocity relation or peak tetanic tension, suggested to these investigators that the site of action of the anions is at or near the excitable surface, not the interior of the fiber. A prolonged period of activation of the muscle, after a single shock, increases the stretch of the series-elastic elements, and an increased isometric twitch tension is measured.

Kahn and Sandow's report prompted the author to make a renewed investigation of the sartorius action potential, which is presumed to reflect surface ionic movements. The results of this investigation are reported below. They show that some of the anions of the lyotropic series produce a clearcut increase in the negative after-potential, the effects increasing in the usual order of the series, $Cl < I < SCN$.

METHODS

Sartorius muscles of *Rana pipiens* were mounted horizontally on a multielectrode assembly (Hill, '49). The muscle was enclosed in a lucite chamber, which was surrounded on three sides by a jacket through which cooled water was circulated. The Ringer fluid around the muscle was kept at 5°C. and covered the muscle, which was raised on the electrode-lucite platform, to a depth of only a few millimeters. The pelvis was fixed, and the tibial tendon connected by a thin straight copper wire to a strain gauge, mounted outside the chamber on a rack and pinion, so that the initial muscle length or tension could be adjusted.

Capillary electrodes, filled with 3 M KCl, were made by the method of Ling and Gerard ('49). The electrode was introduced into the chamber through a small hole in the top and penetrated the surface fibers of the sartorius near the pelvic end. A 954 Acorn tube mounted close to the electrode was used in a cathode follower circuit, and the effective input capacitance, neglecting the electrode, was 1 to 2 uuF. The action potential and its first derivative were displayed on a DuMont 322 double-beam oscilloscope, and photographed.

Measurement of the active state of contraction was made by the method of Macpherson and Wilkie ('54). The response to a single stimulus and then to two closely spaced stimuli was recorded. The two curves were superimposed by double exposure of the photographic film, and the point of divergence of these curves marked the end of the plateau of the active state.

All shocks delivered to the muscle were derived from a Grass 200-B stimulator (1½ msec. square waves), and were supramaximal. The strain gauge (model FT01, Grass Instrument Co., Quincy, Mass.) had a compliance of 2.5 mm per 100 grams. The gauge elements, arranged as a Wheatstone bridge, were supplied with a 2500 cycle per second input signal, and the output signal was amplified, demodulated, filtered and differentiated by circuits of conventional design. Voltages proportional to tension and their first derivatives were displayed on the two-beam oscilloscope.

A solution containing 6.5 gm NaCl, 0.15 gm KCl, and 0.19 gm $CaCl_2 \cdot 2H_2O$ per liter is referred to as Ringer control solution. Solutions containing $NaNO_3$, NaI, or NaSCN, substituted in equimolar quantities for NaCl, were otherwise identical in composition. All solutions contained d-tubocurarine, 1:66,000, 1 millimolar phosphate buffer, and were oxygenated before use. The final pH of all solutions was 7.0 to 7.2.

RESULTS

A. Mechanical measurements

The changes in the mechanical properties of contraction produced by thiocyanate-Ringer are similar to those which have been described for muscles in nitrate, bromide, or iodide-Ringer, but are even more pronounced. The greater the proportion of thiocyanate ion, the more the active state is prolonged. In table 1, the active state plateau duration (measured from the time of the electrical shock) and isometric twitch tension are expressed as a dimensionless *ratio* to the preceding Ringer control measurement. Each muscle was soaked for 15 minutes in the test solution before stimulation, at a temperature of 5°C. and initial tension of 10 to 15 gm. Four muscles were used in each group.

TABLE 1

The active state plateau duration and isometric twitch tension in iodide and thiocyanate-Ringer solutions

SOLUTION	PLATEAU DURATION (dimensionless ratio)	TWITCH TENSION (dimensionless ratio)
100% I	2.3 ± .03[1]	1.41 ± .11
20% SCN	2.4 ± .23	1.32 ± .07
40% SCN	3.0 ± .16	1.40 ± .10
80% SCN	3.7 ± .15	1.45 ± .05

[1] Refers to standard error of the mean.

Although the twitch is potentiated, tetanic tension is nearly unchanged by iodide or thiocyanate substitution for chloride. At 5°C. the active state is normally quite long, and the control twitch : tetanus ratio is about 0.6 to 0.7. In thiocyanate-Ringer, this ratio lies close to 0.9. Because the twitch : tetanus ratio starts at a high value in control Ringer, and cannot exceed 1.0, the twitch ratios listed in table 1 change slowly with marked prolongation of the active state. At higher temperatures, the twitch: tetanus ratio in control Ringer is much smaller, and the increase in twitch tension produced by nitrate or iodide substitution is then striking (Hill and Macpherson, '54).

Table 1 also lists the standard errors of the mean. The greatest contribution to these errors was made by differences from one muscle to the next, rather than variation of the properties of a given muscle from twitch to twitch. The sharpness of the divergence of superimposed twitch and two-shock curves, which determines the accuracy of fixing the end of the active state plateau in Macpherson and Wilkie's method, was a function of the history of the muscle. A series of twitches at one minute intervals clearly showed that the angle of divergence, in thiocyanate-Ringer, becomes progressively smaller.

Several measurements of the active state plateau duration were also made, using a modification of Ritchie's method ('54). They showed that the isometric tension, after the last of a long series of shocks, falls off much more slowly in thiocyanate-Ringer than in control Ringer. A similar effect for muscles in nitrate-Ringer has been described by Ritchie. For this reason, it is difficult to fix the end of the plateau with certainty, and the plateau durations listed in table 1 should be regarded as approximate figures.

The rate at which activated striated muscle shortens is determined by the load on the muscle. This force-velocity relation, described by Hill's equation, can be used to derive the shape of the tension or shortening curves of sartorius muscle under a wide variety of loading conditions, and it is therefore considered as a fundamental mechanical property of the contractile material (Wilkie, '54).

Hill and Macpherson report that iodide and bromide substitution for chloride do not change the force-velocity relation, and the data in figure 1 show that this statement can be extended to include the thiocyanate ion. The method of deriving force-velocity data from isometric tension measurements has been described by Macpherson ('54). The major assumptions implicit in this method are here reiterated:

(1) the instantaneous velocity of shortening, at a given muscle length, is a function only of the tension; (2) the contractile component is in series with an undamped elastic component; (3) forces of inertia may be neglected; (4) the experimental solution does not change the properties of the elastic component.

The relation between the tension (P) in the linear mechanical system and the degree of stretch (x) of the elastic component defines the compliance (c). Because the system is isometric, the increase in length of the elastic component is equal approximately to the decrease in length of the contractile component. The relation $P \cdot c = x$, on differentiation with respect to time, yields $dP/dt \cdot c = dx/dt$. dP/dt is the measured quantity, the rate of rise of tension, c is a parameter which includes non-linear and linear series compliances, and the magnitude of dx/dt is equal both to the velocity of stretching of the elastic component, and to the velocity of shortening of the contractile component. If we do not choose to measure c, because this is difficult to do, we have one relation and two unknown quantities: c and dx/dt. By repeating the experiment

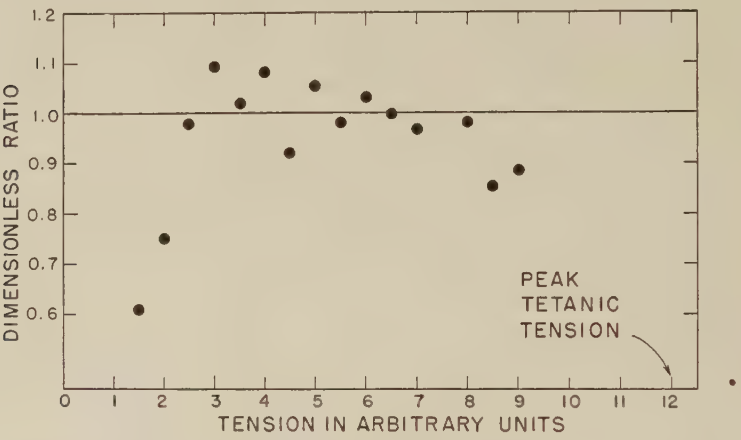

Fig. 1 The ordinate of each point shows the ratio of the shortening velocity in chloride-Ringer to the velocity in 80% thiocyanate-Ringer, for a given tension. Rate of stimulation in chloride-Ringer: 50/sec.; in thiocyanate-Ringer: 20/sec.

with the addition in series of a known compliance, we gain an equation, and can solve for dx/dt.

The muscle from which the data in figure 1 are derived was subjected to 4 successive isometric tetani, the first pair in control Ringer, and the second pair in 80% thiocyanate-Ringer. The second tetanus of each pair was done with an added series compliance, consisting of a small spring attached to the strain gauge. The computed velocity of shortening, at each tension, is expressed on the ordinate as a ratio of velocity in control Ringer to velocity in thiocyanate-Ringer. This ratio was measured for a range of tensions, shown on the abscissa. Several other experiments, using the same procedure, gave comparable results.

If the force-velocity relation were identical in thiocyanate and chloride solutions, if the assumptions were exact, and the experiment perfect, all points would lie on the line 1.0. The deviations from 1.0 at small and large tensions have at least several explanations: (1) the measurement error is considerable at small and large tensions; (2) the spacing of stimuli in control Ringer may have been too great for continuous and complete activation of the contractile component; (3) a true difference in the force-velocity relation at small or large tensions may exist between thiocyanate and chloride-Ringer soaked muscles.

B. Electrical measurements of the resting and action potential

The observations summarized in the introduction to this paper, and the experimental results listed above, suggest that iodide and thiocyanate prolong the active state of contraction by an action at or near the excitable fiber surface. The properties of the contractile material in the fiber interior are probably unchanged. The action potential and its first derivative with respect to time for fibers in iodide and thiocyanate solutions are listed in table 2. The data were taken at 5°C. after a 15 minute soak in test solution. These muscles were at an initial tension of 15 gm so that muscle movement would be minimized. Other experiments at several different initial tensions did not show any significant change in the action potential as a function of length. We may therefore compare these action potential results with the mechanical measurements of table 1. In iodide-Ringer, a distinct increase in the negative after-potential occurs. In thiocyanate-Ringer, this negative after-potential is more pronounced (fig. 2).

TABLE 2

Resting membrane and action potentials in iodide and thiocyanate-Ringer solutions

(The number of fibers averaged is shown in parentheses)

SOLUTION	SPIKE HEIGHT MILLI-VOLTS	RESTING POTENTIAL MILLI-VOLTS	OVER-SHOOT MILLI-VOLTS	AFTER POTENTIAL[1] MILLI-VOLTS	V_1'[1]	V_2'[1]
					(volts per sec.)	
Ringer (7)	130 ± 3[2]	87 ± 3	43 ± 3	8 ± 1.0	96 ± 3	46 ± 2
100% I (9)	132 ± 2	88 ± 1	45 ± 2	25 ± 1.3	54 ± 1	22 ± 1
20% SCN (15)	138 ± 2	88 ± 2	49 ± 2	16 ± 1.8	86 ± 1	35 ± 1
40% SCN (14)	128 ± 3	86 ± 2	41 ± 2	23 ± 2.0	58 ± 3	24 ± 1
60% SCN (11)	128 ± 3	93 ± 3	34 ± 2	31 ± 1.7	58 ± 2	20 ± 1
80% SCN (10)	128 ± 3	97 ± 1	32 ± 2	45 ± 2.2	63 ± 4	17 ± 1
100% SCN (12)	120 ± 2	84 ± 1	35 ± 1	46 ± 1.1	51 ± 2	14 ± 1

[1] The after-potential was measured at 15 milliseconds after the peak of the spike. V_1' and V_2' are the maximum rates of rise and fall of the spike, respectively, obtained by electrical differentiation.

[2] Refers to standard error of the mean.

In figure 3 composite curves are shown representing the average after-potential shapes for muscles in thiocyanate-Ringer solutions. The plateau of depolarization is most pronounced in 80% thiocyanate-Ringer. The after-potential in this solution falls off rapidly about 75 milliseconds after the stimulus. The plateau of the active state of contraction, on the other hand, lasts about 100 milliseconds after the stimulus, and even then does not decline abruptly.

On return to chloride-Ringer after immersion in thiocyanate-Ringer, the muscles again show a small negative after-potential which lasts slightly longer than normal. This failure to revert completely to the initial control level is similar to the behavior of the mechanical properties, which also are incompletely reversible, even after long soaking in chloride-Ringer. The variability in the measurement of the after-potential is sufficiently great so that it is not clear if this incomplete reversibility after thiocyanate-Ringer soaking is real or fortuitous. These data have not been listed. Action potential data for muscles in nitrate-Ringer which show no significant change in the after-potential, have also been omitted.

The measurements of the rate of rise and fall of the spike can be used to derive additional information. If activation of the contractile material is related to the longitudinal current which exists in the fiber core during a propagated action potential, we might expect an increase in the longitudinal current in thiocyanate-Ringer solutions (cf. Bay, Goodall and Szent-Györgyi, '53, and Weber and Portzehl, '54). Table 3 lists in the last column a computed quantity which is proportional to the *maximum* longitudinal current in the fiber core. According to classical cable theory, the longitudinal current (i_2) at any instant of time is given by

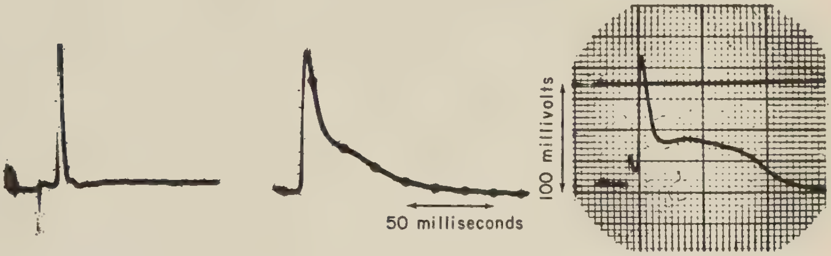

Fig. 2 The action potential on the left is taken from a muscle bathed in normal Ringer. The two on the right, taken at a slightly slower sweep speed, are from fibers in 100% SCN-Ringer. The Z axis modulation in the center photograph is at 60 cps. The resting potential is also shown in the right-hand picture.

On occasion, both of the above forms of the after-potential in SCN-Ringer appeared in adjacent fibers of the same muscle, without transition shapes.

$$i_2 = \frac{-1}{r_2} \cdot \frac{\partial V_2}{\partial x}$$

where r_2 is the resistance per unit length of the axis cylinder in Ω cm^{-1} and $\frac{\partial V_2}{\partial x}$ is the potential gradient in the core along the axis of the fiber. (The choice of symbols corresponds to those used by Hodgkin and Rushton, '46, and Hodgkin and Huxley, '52.) For a muscle immersed in a large volume of fluid, $\frac{\partial V_2}{\partial x} \cong \frac{\partial V_m}{\partial x}$ where V_m is the membrane potential. During steady propagation, $\frac{\partial V_m}{\partial x} = \frac{1}{\theta} \cdot \frac{\partial V_m}{\partial t}$ where Θ is the velocity of propagation.

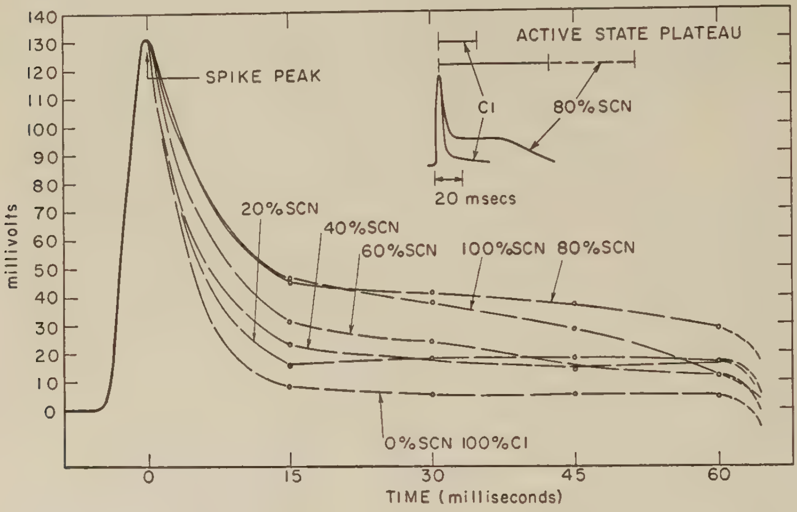

Fig. 3 These curves show the time course of the after-potential, measured at 15, 30, 45, and 60 milliseconds after the spike peak. The spike itself is drawn schematically. The data were taken from the same fibers listed in table 2.

The schematic insert emphasizes the point that the plateau of the active state in thiocyanate-Ringer is longer than the plateau of depolarization of the after-potential.

Then $i_2 \cong \frac{-1}{r_2\theta} \cdot \frac{\partial V_m}{\partial t}$. If we assume that the resistance of the axis cylinder, which is largely determined by the electrolyte content of the muscle fiber and the fiber diameter, is not appreciably changed by brief immersion in thiocyanate-Ringer, then we need only the velocity of propagation and the first derivative of the action potential with respect to time for the computation. The muscle was bathed first in

Ringer control solution, and then for 15 minutes in thiocyanate-Ringer. The velocity of propagation was measured by stimulating the muscle near the tibial end, and recording the action potential 3 cm away at the pelvic end, with an intracellular electrode.

The decrease in the maximum rate of rise of the action potential, in thiocyanate-Ringer, is similar to the results recorded in table 2. The velocity of propagation in thiocyanate-Ringer is not significantly different from that in control Ringer. The computed peak longitudinal current, then, is not larger, but 30% smaller in thiocyanate-Ringer.

TABLE 3

Maximum longitudinal potential gradient in the fiber core during propagation

(The number of fibers averaged is shown in parentheses)

SOLUTION	SPIKE HEIGHT MILLIVOLTS	$\frac{\partial V_m}{\partial t}$[1] (max)	θ	$\left\{ \frac{1}{\theta} \cdot \frac{\partial V_m}{\partial t} (\text{max}) \right\}$
		. . . . . . units arbitrary		
Ringer (5)	138 ± 4 [2]	14.6 ± .2	10.7 ± .4	1.36 ± .04 [3]
80% SCN (7)	131 ± 3	10.7 ± .3	11.2 ± .2	0.96 ± .11

[1] $\frac{\partial V_m}{\partial t}$ in this table and V_1' in table 2 were determined in the same way.

[2] Refers to standard error of the mean.

[3] Computed for each fiber before taking the mean.

DISCUSSION

Substitution of thiocyanate ion for chloride in Ringer solution produces two readily observable changes in frog sartorius muscles at 5°C.: (1) potentiation of the twitch, and (2) an increase in the duration of depolarization during the action potential — i.e. an increase in the negative after-potential. In iodide-Ringer, these changes are less marked. In nitrate-Ringer at low temperatures, prolongation of the active state is even smaller, and the change in the after-potential is absent or too small for easy detection. The implications of the mechanical measurements in nitrate or iodide solutions have been discussed in detail by several investigators (loc. cit.).

These anions appear to act on the fiber surface, prolonging the active state of contraction but leaving nearly unchanged the force-velocity relation and the tetanic tension.

The experiments on muscles in thiocyanate-Ringer, reported in this paper, suggest that the duration of the active state of contraction is linked to the repolarization process. There is accessory evidence, in part summarized by Katz ('50) which favors this interpretation for striated muscle. For example, substitution of tetraethylammonium for sodium ion in the medium bathing crab muscle causes a large increase in both amplitude and duration of the electrical and mechanical response (Fatt and Katz, '52). On the other hand, the active state is prolonged in nitrate-Ringer without a clearly detectable change in the action potential (Kahn and Sandow, '50, and this paper). The action potential, however, is a potpourri of an unknown number of ionic currents. A small but important change in the ionic transference numbers for a muscle in nitrate-Ringer might go undetected in an action potential measurement.

The action potential of single fibers in thiocyanate-Ringer, which has not been described before, resembles in some respects the action potential in cardiac muscle fibers. Weidmann ('51) has shown that during the plateau of depolarization in mammalian Purkinje fibers, the membrane resistance is higher than during repolarization. This suggested that the delayed repolarization was caused by a wide separation in time of the sodium influx (the rising phase of the spike) and the potassium efflux (repolarization).

The repolarizing process in thiocyanate-Ringer, which proceeds in at least two steps, needs clarification. If we suppose that the repolarizing current is carried only by potassium ions, then we must explain why this repolarizing process is interrupted. An alternative hypothesis is that the two phases of repolarization represent the flux of two different ion species. For example, the initial step of about 80 millivolts may reflect a net inward flow of thiocyanate ions, and the final step a delayed outward flow of potassium ions. Both thiocyanate and potassium ions would flow from a region of higher to a region of lower electrochemical potential. These alternatives can be tested experimentally if a method is developed for resolving ion fluxes during activity into their component temporal parts.

SUMMARY

1. A correlative study of the active state of contraction, and the resting and action potential in frog sartorius muscle fibers at 5°C. is described. When the muscle is bathed in Ringer solution containing thiocyanate ion substituted for chloride, the following is observed:

(a) The duration of the plateau of the active state of contraction is increased almost fourfold.

(b) The force-velocity relation, and the maximum force in a tetanus, are not appreciably changed.

(c) The negative after-potential is markedly increased to 35 or 40 millivolts, and lasts about three times as long as the main spike.

(d) The maximum rate of fall of the spike in thiocyanate-Ringer is about one-third that in chloride-Ringer.

(e) The maximum longitudinal current in the fiber core during a propagated impulse is about 30% smaller in thiocyanate-Ringer than in chloride-Ringer. This decrease is largely due to a decreased rate of rise of the spike. The conduction velocity is unchanged.

2. The changes in the active state, and the after-potential, produced by anion substitution, are arranged in the usual order of the lyotropic series: $Cl < I < SCN$.

ACKNOWLEDGMENTS

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ADDENDUM

The following relevant papers, which appeared after this paper had been submitted, should be consulted: Harris, E. J., and H. Martins-Ferreira 1955 Membrane potentials in the muscles of the South American frog, Leptodactylus Ocelatus. J. Exper. Biol., *32:* 539–546; Kahn, A. J., and A. Sandow 1955 Effects of bromide, nitrate, and iodide on responses of skeletal muscle. Ann. N. Y. Acad. Sci., *62:* 137–176.

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TRANSTUBULAR POTENTIAL DIFFERENCES OF RAT KIDNEY

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FIVE FIGURES

INTRODUCTION

Studies of electrochemical activity across cell membranes and across secretory cell sheets have been found to be of use in elucidating mechanisms involved in solute transfer. Thus it has been shown that the difference in electrical potential across frog skin arises as a consequence of sodium transport (Huf '35; '53; Ussing and Zerahn '51; Linderholm '52). Also the potential difference across the gastric mucosa seems to be a consequence of chloride transport (Hogben '51, '55), and the electrical activity of nerve arises through consecutive movement of sodium and potassium across the cell membrane (Hodgkin and Huxley '52). With regard to the kidney tubule, only one report has been found wherein renal electrical properties have been investigated (Wilbrandt '38). It would seem that the use of microelectrodes such as have been utilized in studying transmembrane potentials of muscle (Graham and Gerard '46; Ling and Gerard '48) would lend itself to investigation not only of trans-tubular potentials, but also of transmembrane electrical activity of the renal secretory epithelium. This paper reports on certain properties of the transtubular electrical potential of the rat.

METHODS

The preparation of animals in most part followed the methods of Walker and Oliver ('41). Male rats, generally weighing over 400 gms. were used in these experiments. All animals were deprived of food 16 hours prior to experimentation. Some of these were allowed free access to drinking water (referred to as hydrated animals); while others were deprived of water also for a 16 hour time period (dehydrated animals). Each rat was anaesthetized with nembutal (30 mg./-kg.), pinned to a board, and a midline incision running the full length of the abdominal cavity was made. The abdominal walls were then fixed latterally and downward by fishhooks attached to T pins. The viscerea were moved to the left side of the abdominal cavity and the right kidney blood vessels and the ureter were ligated at the renal pedicle. The viscera were then moved to the right side of the abdominal cavity and fixed in place with pins pushed through the dorsal body wall. Pins also held the liver, pancreas, and spleen from pushing against the left kidney so as to reduce kidney movement because of respiration. Exposed viscera were covered with moist absorbent cotton and the whole abdominal cavity was filled with Ringers solution. Under such conditions, however, the kidney still shows a great deal of movement as a consequence of respiratory efforts and pulse transmission (Walker and Oliver '41). To minimize this difficulty, a lucite ring of one inch outside diameter and 1/2 inch inside diameter was tied to pins pushed through the body walls lateral to the kidney. Downward pressure exerted with the ties reduced movement. It has not been possible, however, to eliminate all movement in every animal without some apparent reduction of blood flow caused by compression of blood vessels. Reasonable working conditions can always be established, however, in that the amount of movement can always be minimized to the extent that proper penetration into tubles is not seriously hampered. The board was then placed on the stage of a Spencer binocular microscope, and the kidney illuminated by a microscope lamp which also served to keep the abdominal contents warm. Temperatures were maintained at 34°—36° C. in this way. Observations were made at magnifications of either 90x or 120x.

Microelectrodes were prepared according to the method of Ling and Gerard ('48), but were filled with 3M KCL (Nastuk and Hodgkins '50). A single electrode was mounted in a glass holder which was connected to a Beckman calomel half cell. This, in turn, fed to an electrometer tube (Victoreen 5803) cathode follower of conventional design, which, in turn, led to a D.C. amplifier (Moore '49). The peritoneal saline solution was grounded through a second calomel cell to complete the circuit.

The electrode holders were mounted in one of two micromanipulators secured to a large steel plate. One of these was a commercial device. The other was made in this laboratory. It consisted essentially of two racks and pinions from discarded microscopes mounted at right angles to each other, with the vertical rack and pinion supporting the manipulator head. This arrangement allowed for up-down and left-right movements of a relatively fine degree. Back-forth movements were done manually, since it was found that for the purpose of these experiments, such was adequate.

A word is in order about the choice of electrode used in the experiments to be described. One is faced with overcoming two problems. Because of the presence of the capsule penetration is difficult. It seemed to be most easily accomplished by electrodes with relatively short sharp tapering tips. On the other hand, because of residual movement, breakage of electrodes with this shape is so great as to become almost prohibitive to carrying out an experiment. As might be expected, it was found that electrodes with long thin tips would last longer and allow for prolonged maintenance of deflections. For these reasons, thin electrodes were preferentially used despite penetration problems.

RESULTS

If one proceeds to insert a microelectrode at random into various tubules, it is found that the potential difference values (P.D.) fall into two distinct groups. Such bimodal distributions, as shown in figures 1 and 4, were found in all but one experiment wherein a wide continuous distribution was obtained.

In figure 1 the two top histograms show the results of two individual experiments which are typical of the extremes in variation which are obtained. In the top histogram the division between potential populations is immediately apparent while in the lower experiment it may be doubtful.

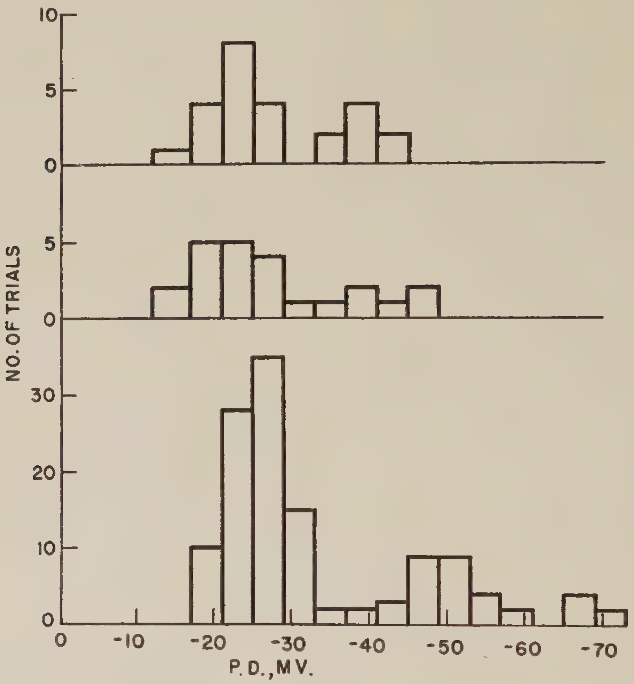

Fig. 1 Distribution of P.D. obtained from randomly punctured tubules. Upper histograms are from individual experiments. Lower histogram shows pooled data from six experiments.

The lowest histogram of figure 1 shows the distribution which results from pooling of six experiments wherein comparable values for transtubular P.D. were obtained. As might be expected, pooling of the data sharpens the two peaks and brings out to a greater extent the bimodal distribution.

Means of the two distributions of a group of representative experiments are given in table 1. The range for the lower valued distributions is 19-39 mv. with the inside of the tubule negative to the outside. The higher valued distributions range from 34-70 mv. with the inside of the tubule again being negative. It is also seen that the P.D. across the tubules of dehydrated animals are in general higher than that obtained from hydrated animals. On some few occasions, much higher negative values, on the order of 90-100 mv., have been obtained. Since they are so infrequent, and since they are of the order of magnitude found for the transmembrane potential of nerve and muscle (Huxley and Stampfli '51, Ling and Gerard '48) it is suspected, although not proven, that these values are representative of transmembrane potentials of the secretory epithelium. Moreover, it has also been observed that during tubular penetration, a deflection of higher value than the one which is finally obtained, is frequently observed. Such finding is consistent with the idea of the electrode passing through cells of greater electrical negativity than that of the lumen. Because of the original high deflection, one is forced to consider at what time the galvanometer should be read. After

TABLE 1

Mean values of low and high population of randomly punctured kidney tubules. Each line represents values obtained from a single animal, N giving the number contributing to each mean. Means are given in millivolts

Transtubular potentials

N	LOW MEAN ± S.E.	N	HIGH MEAN ± S.E.
30	28 ± 1	1	54 ± 3
16	27 ± 1	10	39 ± 1
16	20 ± 1	6	40 ± 2
14	19 ± 1	5	43 ± 2
17	20 ± 1	8	37 ± 1
11	23 ± 1	3	37
12	20 ± 2	2	39
11	29 ± 2 [1]	5	61 ± 4 [1]
20	30 ± 1 [1]	4	45 [1]
15	39 ± 1 [1]	6	70 ± 3 [1]
16	29 ± 1 [1]	5	59 ± 3 [1]
14	28 ± 2 [1]	6	49 ± 3 [1]

[1] Dehydrated animals.

being satisfied that the electrode tip was in the tubule lumen, that potential difference which could be maintained at a steady value until the electrode was dislodged accidently or removed, was taken as the value for that particular transtubular P.D. Steady deflections were always established in a few seconds, and readings were generally made at about five seconds after entry. Insomuch as visual observation of the puncture procedure is valid for establishing the position of the electrode tip, the potentials obtained are taken to represent the transtubular P.D.

TABLE 2

Comparison of potential distributions in kidneys with the capsule on and then subsequently removed

CAPSULATED				DECAPSULATED			
Low P.D.s		High P.D.s		Low P.D.s		High P.D.s	
N	Mean	N	Mean	N	Mean	N	Mean
9	21	3	33	7	22	5	38
6	21	7	35	12	22	4	35
10	23	7	47	9	21	8	45
8	29	8	51	7	26	7	50
6	22	9	36	7	23	9	38

Since connective tissue capsules can act as barriers to diffusion (see Feng and Gerard, '30, for the case of the nerve sheaths), it may be argued that the grounding of a solution external to the capsule does not allow for proper measurement of the transtubular potential as the capsule may constitute an electrical resistance which cannot be neglected. To evaluate this possibility, a direct attack on the problem appeared best. After a suitable number of control readings, the kidney capsule was stripped and again P.D. values were determined. The data are given in table 2, and it is seen that little difference exists between mean values before and after decapsulation. In no case was there a significant change. Walker and Oliver ('41) found that decapsulation led to an altered kidney histology, the cells becoming granular and opaque in about 20 minutes following decapsulation. Their observations were confirmed in these experiments, but it was noted that such treatment did not seem to interfere markedly with electrical activity. The potential differences were maintained for as much as an hour following decapsulation.

The question arises whether the values obtained are of biological significance. One would expect that if these potentials are biologically important that (1) they can be maintained for extended periods of time, and (2) that interference with the functioning of the tubular cells would result in changes in their values. Figure 2 shows an experiment in which a microelectrode was inserted into the tubule lumen and allowed to remain in position. Relatively little change occurs until 4.5 minutes after insertion, whereupon the animal made a deeper than usual respiratory effort. This resulted in some electrode displacement as shown by the fall-off in value at this time, but the electrode apparently returned to a favorable position since the fall was reversible. At 8′ 45″, the animal gave a second gasp, and this time the electrode was either permanently dislodged, or more likely, a relatively large hole was made in the tubule, since the potential value does not fall to zero, but rather to the low value of 11 mv.

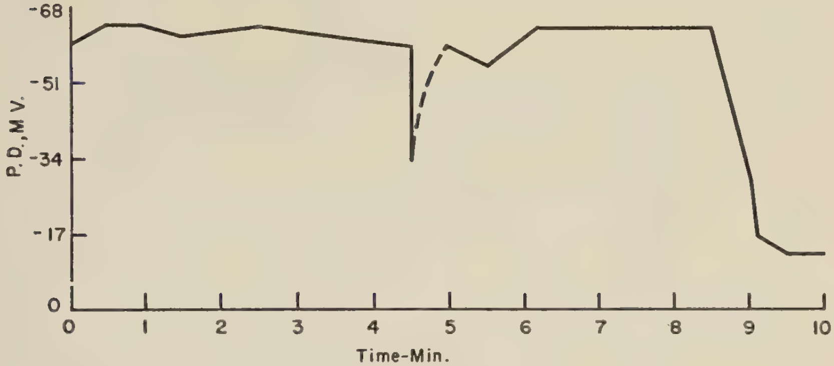

Fig. 2 Potential difference across a single tubule shown as a function of time. A broken line is shown between 4.5 and 5.0 minutes as the exact time course was not followed. For complete explanation see text.

Figure 3 shows the results of three experiments wherein the renal artery and vein were clamped for a period of ten minutes. It is seen that during the clamped period there is a fall-off of potential values to almost zero, and that this depression is fully reversible in a short time after re-establishment of blood flow.

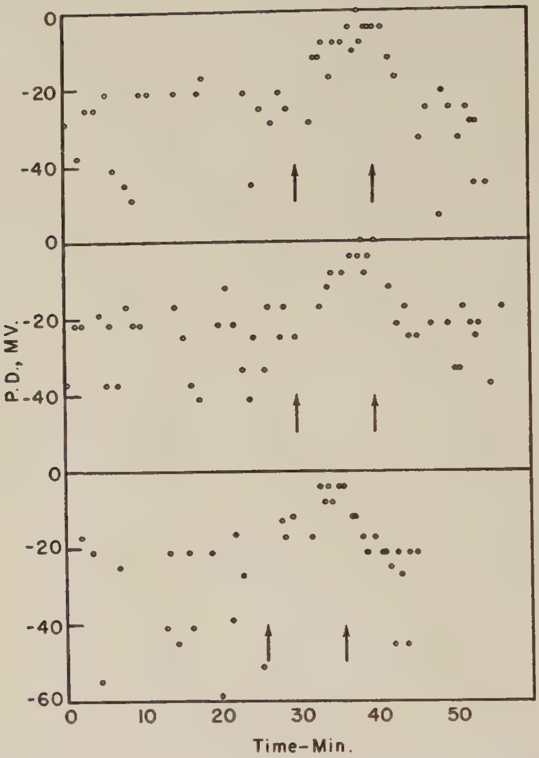

Fig. 3 Effect of circulatory interruption on transtubular P.D. Renal artery and vain were clamped for the time period bracketed by the two arrows in each experiment.

One would expect on the basis of the morphology of the mammalian kidney that two distributions of potentials would be found, one for proximal tubules and one for distal tubules. Since proximal tubule segments are more frequent at the surface than distal tubules, inspection of the previous data would suggest that the lower potential values are a consequence of electrochemical differences across the proximal tubule, with the higher values coming from the distal tubules. In order to test this hypothesis, phenol red was used for the identification of the two tubular segments (Walker and Oliver '41). After a control period, six mg./kilo of phenol red (dissolved in Ringers solution at a concentration of six mg./c.c.) was injected through the jugular vein and transtubular measurements were again taken. Those tubules which were of deeper color than others were considered to be distal. Since the dosage of dye injected was relatively high, differentiation between proximal and distal tubular segments was quite difficult immediately following injection. As a consequence, there were probably some errors in identification. Figure 4 shows a comparison of control potential values with the

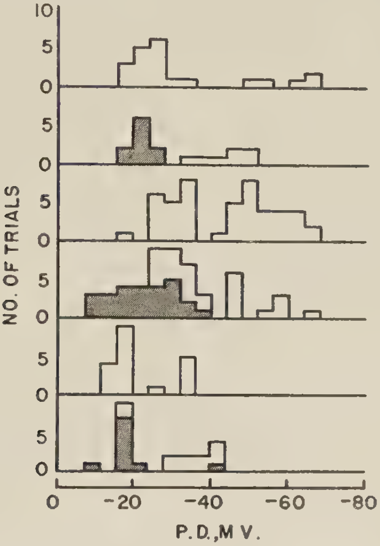

Fig. 4 Comparison of P.D. of randomly punctured tubules with phenol red derived P.D. across proximal and distal tubules. Upper clear histograms of each pair show control distributions. In lower histograms, cross hatched areas show values obtained from proximal tubules, clear areas show values obtained from distal tubules.

TABLE 3

Comparison of control distribution and Phenol red derived proximal and distal tubular distributions of potential differences

CONTROL				AFTER PHENOL RED			
Low		High		Proximal		Distal	
N	Mean ± S.E.	N	Mean ± S.E.	N	Mean ± S.E.	N	Mean ± S.E.
14	28 ± 2	6	49 ± 3	16	21 ± 1	1	49
20	33 ± 1	28	57 ± 1	27	24 ± 2	29	42 ± 3
14	20 ± 1	4	34	7	16 ± 2	4	29 ± 3
15	23 ± 1	11	43 ± 3	13	27 ± 4	16	45 ± 1
15	21 ± 1	8	40 ± 3	13	17 ± 1	11	32 ± 2
14	20 ± 1	5	37 ± [1]	10	23 ± 1	11	37 ± 3
14	27 ± 1	5	46 ± 4	7	24 ± 1	5	43 ± 1
11	29 ± 1	5	61 ± 4	10	25 ± 2	7	47 ± 3
16	29 ± 1	5	59 ± 3	8	29 ± 1	8	52 ± 4

[1] All measurements were identical.

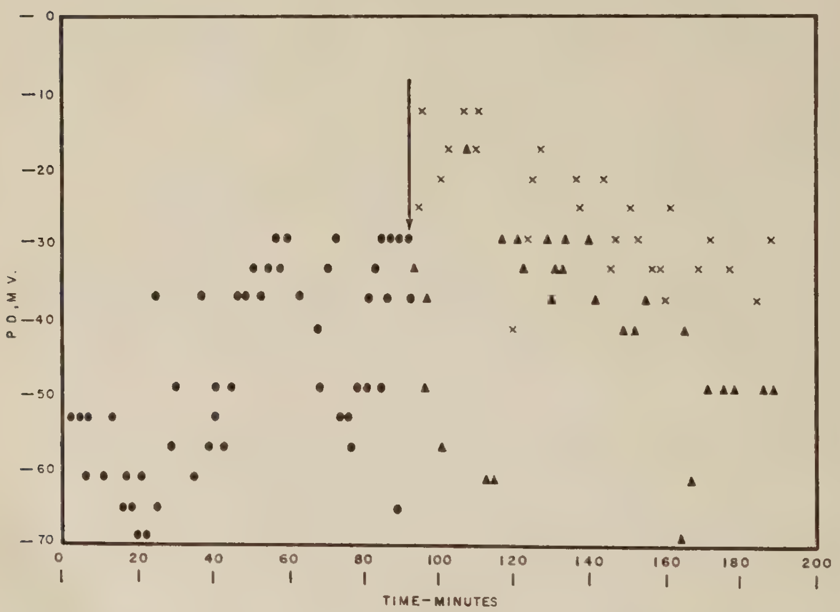

Fig. 5 Experiment showing the effect of phenol red on transtubular P.D. Control period given by ●; proximal measurements by ×; and distal mearurements by ▲. Dye injected at the arrow.

distribution of proximal and distal values obtained after phenol red injection in three experiments. Table 3 summarizes the results of all experiments thus far carried out.

It can be seen that the low potential distribution of the control period approximates the electrochemical activity difference found for the proximal tubules, with the high distribution approximating the P.D. across the distal tubules. This is only an approximation for two reasons. Because of probable errors made immediately after injection as mentioned above, there could occur overlap of proximal and distal values. The three values which appear to be misplaced in the lower experiment of fig. 4 may be explained in this manner, since these particular results were obtained within five minutes after injection. A second source of overlap comes from the fact that a slight to moderate fall in P.D. may occur following the injection of phenol red. This error is shown in figure 5, wherein the potential values as a function of time in the randomly punctured tubules for the middle experiment of figure 5 are plotted. From the values obtained along with the above considerations, it is tentatively concluded that the lower potential differences exist across the proximal tubules, while the higher values are across distal tubules.

DISCUSSION

In considering the results presented, the question arises as to whether the data obtained under the conditions of these experiments in any way reflect events which occur in the intact animal. With the rather extensive surgery and moderate levels of anaesthesia, it may be argued that the state of the animal is such that the data have little validity as far as normal kidney function is concerned. It would be premature to attempt to evaluate the nature of any differences between animals under these conditions and in the non-operated unanaesthetized state. One might expect oliguria as a consequence of the surgical trauma and anaesthesia (Smith '52); however, rats under these conditions do produce urine at rates of from 20–90 mm^3/min/square meter surface area.

To arrive at any conclusions concerning the underlying mechanisms which give rise to the transtubular potentials would be premature on the basis of the data here presented. The fact that several individual transport systems exist for different urinary constituents would suggest that the final number which one gets for the potential difference may be a consequence of several electrochemical potential gradients for various substances. Moreover, several mechanisms possibly exist as to the origins of the gradients. Of these it would appear that two seem most likely. One could be measuring the resultant of several passive diffusion potentials; or as in the case of frog skin (Linderholm, '52; Huf and Wills, '53), the potential could be a reflection of the transport activity of the secretory cell sheet. At any event, it is dependent upon an intact blood flow to the kidney, and one may therefore conclude upon some type of function being maintained in the kidney.

In the course of these experiments, the assumption has been made that the transtubular potential gradient exists between tubular contents and interstitial fluid. It may be argued by some that tubular activity is directed between blood stream and tubular contents and that therefore these measurements are not the most interesting P.D. measurements which may be made. This argument requires that each tubule cell be in contact with a capillary, but the histology of the kidney does not seem to show that this is the case. Moreover, Kaplan, Friedman and Kruger ('43) have found that the renal lymph is derived in part from the blood and in part from fluid reabsorbed by the tubules. Since lymph is derived from the fluid surrounding cells, their experiments suggest that tubular activity is not directed only between blood and lumen, and may be taken as evidence in support of the idea that the biologically meaningful P.D. exists between lumen and interstitial fluid.

Willbrandt ('37) found that in Necturus the inside of proximal tubule is negative to the outside and with the distal tubule the sign is reversed. In the experiment described here, the insides of both proximal and distal tubules were found to be negative. This need not be a contradiction in results, since two quite different species of animals were used, and the processes involved in potential generation may also be quite different.

In the case of the phenol red results, one is faced with the difficulty of explaining the drop in the potential differences across the tubules subsequent to treatment. One would hardly expect any change to occur since at the pH range which can be found in urine most of the phenol red present is uncharged. Also, if the dye were present in an ionized form, it would be an anion and the net effect of secretion would be to add negative charges to the luminar contents. If such were possible, an increase in P.D. would be expected. The fall that occurs is probably not a result of the above considerations at all but comes from wholly different reasons. In studies now in progress, it has been found that the transtubular potential of both proximal and distal tubules is related to the rate of urine flow, in that as urinary volume increases, potential falls. Although urine volumes were not measured in the present particular experiments, it is quite likely that the rate of urine production increased postinjection, because of (1) increased urinary output resulting from the excretion of the injected Ringers solution which served as carrier for the dye, and (2) the diuretic effect resulting from the increase in osmotic pressure attendant on the secretion of the dye. It is therefore suggested that the occasional fall in potential resulting from phenol red treatment is dependent upon the diuretic effect of such treatment and not on the secretion of the dye *per se*.

SUMMARY

1. Measurements of the potential differences across the tubules of rat kidney have been made. The distribution of values obtained is bimodal, with the lower means ranging from 19-39 mv., and the higher means ranging from 34-70.

2. Decapsulation of the kidney does not lead to any change in P.D.

3. The P.D. can be maintained for an extended period of time.

4. Cutting off blood flow leads to a fall in P.D. to low values, the fall being reversed with the reestablishment of blood flow.

5. By the use of phenol red to indicate proximal and distal tubules, evidence is presented to show that the low P.D. results from the electrical activity across the proximal tubule, whereas the high P.D. results from activity across the distal tubule.

6. Treatment with phenol red may cause the transtubular P.D. to fall. It is suggested that the fall in potential is a consequence of the increased urinary output which accompanies phenol red treatment.

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METABOLISM OF HELA CELLS: METHODOLOGY AND MEDIA EVALUATION[1]

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THREE FIGURES

Strain HeLa cells, derived from an epidermoid cervical carcinoma (Gey, '52; Scherer et al., '53) are currently employed in this laboratory in studies of the physiology and biochemistry of poliovirus replication in animal cells. The use of manometry for determination of the metabolitic acivity of HeLa cells has been reported (Gifford et al., '54). This communication relates to the application of manometric procedure to: (a) estimation of cellular growth, and (b) evaluation of some complex media and buffers for use on HeLa cell cultures.

MATERIALS AND METHODS

Cultivation of cells. Strain HeLa cells were propagated in quantity in planar screw-capped 200 ml bottles in a medium of 40% human serum and 60% Hanks's balanced salt solution (HuS-40, H-60), incubated horizontally at 35°C. The cultivated cells were washed three times, dispersed for transfer or other purposes by exposure to 0.5% trypsin (Difco, 1 : 250) in H-100 (pH 7.6) for 30 to 60 minutes at room temperature. After trypsinization the resultant uniform suspension of single cells or small clumps was washed once in H-100 and suspended in test medium or in H-100 for transfer to sterile

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Warburg flasks. The details of cell cultivation, maintenance and enumeration have been reported by Syverton and Scherer ('54).

Media. Human serum (HuS-100) from blood drawn aseptically from healthy donors was pooled and stored at 3 to 7°C. Chicken serum (ChS-100) in quantity was separated from blood obtained at an abattoir, sterilized by passage through Selas micro-porous porcelain candles 02 and 01 porosity, and stored at 3 to 7°C. Maintenance solution (MS-100) was prepared as reported by Scherer ('53). Modified maintenance solution (MMS-100) was prepared by omission of all components of MS-100 present at a concentration of less than 5 mg per liter and by elevation of the bicarbonate concentration from 0.035 to 0.056%. Medium 703 was prepared according to the formula of Healy, Fisher and Parker ('54). Penicillin and streptomycin were added to media in respective concentrations of 300 units and 100 μg per milliliter. Desiccated yeast extract (Difco) was dissolved in MMS-100 at a final concentration of 0.1% and the pH adjusted to 7.6 with sodium hydroxide; the resultant medium (YEM-100) was sterilized by sintered glass filtration.

Buffers. Sodium hydrogen phosphite solution (BPS-100) was prepared as previously reported (Gifford et al., '54) to provide a final phosphite concentration of 0.02 M. Sodium hydrogen phosphate, sodium barbital and tris (hydroxymethyl) amino methane (THAM) were prepared as isotonic solutions in Hanks's balanced salt solution with an equivalent amount of sodium chloride omitted. The pH was adjusted to 7.6 with sodium hydroxide. The usual amount of sodium bicarbonate was added to the buffer solutions, since preliminary studies indicated that without added bicarbonate, buffer capacity at desired pH levels was inadequate for growing cell cultures. Buffer solutions were sterilized by sintered-glass filtration.

Manometric method. Oxygen consumption was measured by use of Krebs's ('51) modification of Pardee's carbon dioxide buffer at a concentration providing at equilibrium 2% carbon dioxide in the gas phase. Amounts of 0.5 ml of buffer were placed in: (a) flask center wells fitted with filter paper wicks, and (b) flask side arms. A convenient rate of respiration was found with from 400,000 to 2,000,000 HeLa cells per flask. Flasks were gassed for one hour with 2 (± 0.5) per cent carbon dioxide in air while shaken 60 times per minute with an amplitude of 2.5 cm. The flask cultures were incubated at 35°C. and respiration measured in the conventional manner. HeLa cells in the agitated flasks assumed a position at the gas-liquid-glass interfaces.

RESULTS

Effect of varying cell inocula on oxygen consumption and replication rate of strain HeLa. Various numbers of HeLa cells were added to duplicate Warburg flasks and oxygen consumption was measured for 68.5 hours. The results (table 1) indicated that: (a) the rate of oxygen consumption was related linearly to number of both cells inoculated and cells harvested;

TABLE 1

Effect of varying cell number on the oxygen consumption and replication rate of strain HeLa

INITIAL CELL NUMBER IN THOUSANDS	FINAL CELL NUMBER IN THOUSANDS	OXYGEN CONSUMPTION		PER CENT MULTIPLICATION OF CELLS (calculated)	CALCULATED LIVE CELLS AT START IN THOUSANDS	PER CENT SURVIVAL AFTER DISPERSION
		Start	End			
		μl/hr.				
6800	3320	28.2	30.0	6	3121	46
6800	3320	26.0	27.8	7	3088	47
4200	2900	18.9	24.4	29	2246	53
4200	2840	18.9	24.0	27	2236	53
3400	2430	12.2	19.1	57	1449	43
3400	2600	13.6	19.3	42	1832	54
1700	1500	6.0	12.2	103	738	43
1700	1520	6.4	12.2	91	797	47
850	800	3.6	8.2	128	351	41
850	800	3.8	8.4	121	362	43
470	506	2.0	5.8	190	174	37
470	650	2.7	5.6	107	313	67

and (b) within limits the rate of oxygen consumption increased with time with the net effect inversely proportional to inoculum size. Although the manometric data suggest varying cell multiplication during incubation in the flasks, a comparison of cell numbers harvested with cells inoculated (table 1) indicated net reductions in population in most cases. The apparent paradox was considered to result from the presence of dead cells in the counted inocula, since previous experiments by one of us (J.T.S.) had showed that about half of trypsin-treated populations were not viable. Estimates of the number of viable cells inoculated were calculated from the manometric data, on the assumptions that only viable cells remained attached to the flask walls after long incubation, and that the rate of increase in oxygen-consumption rate was proportional to the increase in cell population. The agreement of survival rate estimates (table 1) despite wide differences in calculated rates of multiplication is evidence for the validity of the assumptions. It is seen that cellular multiplication with incubation was insignificant when large cell inocula were employed indicating a maximum population.

Effect of medium serum concentration on respiration of HeLa cells. For quantitative studies of viral replication two types of medium are needed: (a) an optimum medium which is simple, economical and readily prepared for cell cultivation, and (b) a maintenance antibody-free medium permitting maximum metabolic activity of cells without net multiplication. The relation previously shown (Gifford et al., '54) between cellular respiration and viral replication suggested that medium (a) should promote maximum respiration as well as adequate multiplication of cells. The effect of human serum concentration on adequacy of the conventional growth medium (HuS-40, H-60) was evaluated manometrically over an incubation period of 72 hours. Figure 1 summarizes the relation between respiration, serum concentration and time. The stimulation of respiration of HeLa cell cultures with increasing concentrations of human serum reached a maximum at 40% by volume. Serum in concentrations of 10 and 20% resulted in maintenance of cells as shown by an almost constant rate of oxygen uptake with time. Serum in concentrations of 30, 40 and 50% resulted in growth as shown by the increasing rate of oxygen uptake with time.

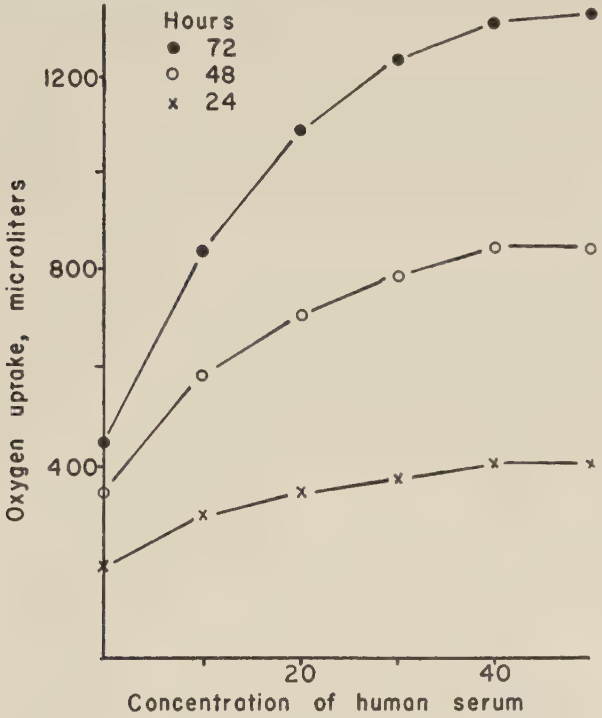

Fig. 1 Relation between oxygen uptake, human serum concentration and time.

The influence of chicken serum concentrations on cellular respiration was determined with Hanks's solution containing 10 or 20% serum. The former concentration commonly is employed in media used for maintenance of HeLa cells. The rate of oxygen uptake of an initial cell population of 1 million cells was 10 µl per hour. At the end of 85 hours of incubation the rate of respiration in 10 and 20% chicken serum was 11 and 14 µl per hour respectively. The respective rates of change of oxygen consumption with time showed that 10% chicken serum was satisfactory for maintenance while the higher concentration allowed some multiplication.

Effect of added glucose. It was possible that the increase in respiration with increased serum resulted from the higher glucose concentration. Aliquots of 2,000,000 HeLa cells in ChS-10, MMS-90 were dispensed into Warburg flasks containing 0, 1.5 or 3.0 mg per milliliter of added glucose. The added glucose up to 3.0 mg per milliliter did not alter significantly the rate of respiration of HeLa cells incubated for 49 hours, but seemed to sustain the respiratory rate over longer periods of incubation. After 49 hours the cells without added glucose failed to maintain whereas in the presence of glucose maintenance continued for an additional 25 hours. A concentration of glucose, 1.5 mg/ml was found superior to 3.0 mg/ml.

Effect of various media on maintenance of HeLa cells. For virus studies, the ideal maintenance medium should maintain cellular integrity, metabolic activity and morphology in the absence of multiplication, for 7 or more days. Various media which are or could be used for the maintenance of HeLa cells were examined for their effect upon respiration. Cells grown in HuS-40, H-60 were dispersed with trypsin, washed and resuspended in maintenance medium. Aliquots containing about 500,000 cells were dispensed into Warburg flasks and the respiration followed for 85 hours. Two commonly employed media, ChS-10, H-90 and ChS-10, MS-90 were tested first. The bicarbonate concentration of Hanks's solution was increased from the usual 0.035 to 0.056%, to compensate for the lower buffer capacity compared with that of MS. The rate of oxygen consumption (10 μl/hour/million cells) remained essentially constant with either medium throughout the 76 hour period of observation and each affected respiration similarly. The rather poor buffering action of ChS-10, H-90, even in the presence of added bicrabonate, led to the replacement of Hanks's solution with modified maintenance solution in many experiments.

Parker's synthetic medium 703, recently developed for growth of strain L cells, was tested similarly in comparison with HuS-10, H-90 medium, ChS-10, H-90 medium, and H-100 alone. The course of oxygen consumption in the presence of these media is shown in figure 2. With both 703 and H-100 the decreasing rate of cellular respiration indicated unsatisfactory maintenance. As expected, HuS-10, H-90 stimulated respiration to a greater extent than did ChS-10, H-90.

Effect of yeast extract on respiration of strain HeLa. HeLa cells in MMS-100 were dispensed into Warburg flasks and 4 flasks each made to contain 0, 10 or 40% of human serum by volume. A solution of yeast extract (Difco) was added to 2 flasks at each serum concentration so that the final concentration was 0.1%; instead of yeast extract, additional MMS was added to the other pair of flasks at each serum concentration. The results (fig. 3) indicated that yeast extract regularly enhanced the respiration of cultures both in the presence and in

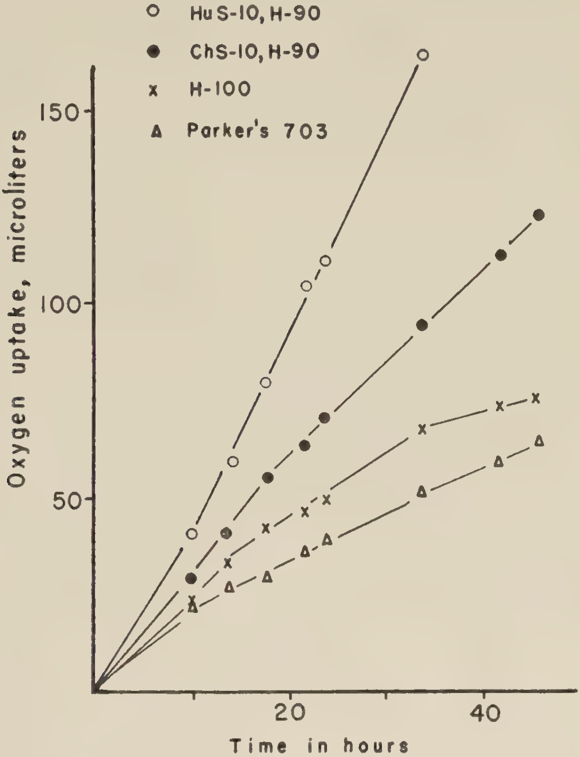

Fig. 2 Effect of synthetic medium no. 703 (Parker), balanced salt solution (Hanks's) and serum on respiration of strain HeLa.

the absence of serum. The serum sparing effect was apparent: cells in HuS-10, MMS-90 with 0.1% yeast extract respired at essentially the same rate as cells in HuS-40, MMS-60 without yeast extract.

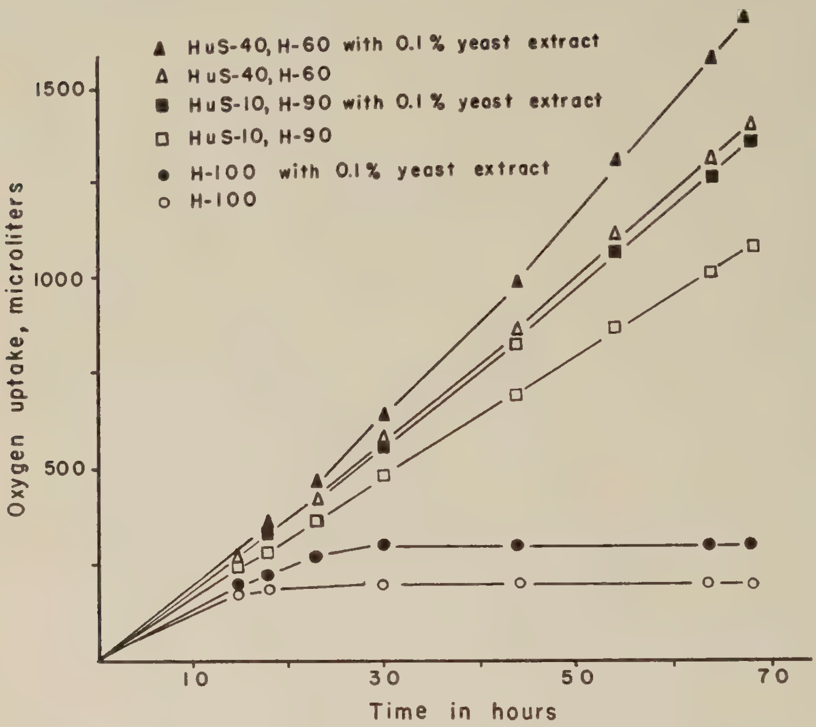

Fig. 3 The effect of yeast extract upon respiration of strain HeLa cells.

Effect of buffers on HeLa cells. Isotonic solutions of buffers in ChS-10, H-90 were prepared and tested for effect on respiration of HeLa cells. Table 2 lists the oxygen consumption of HeLa cells at 24, 53 and 78 hours after suspension in 0.02 M sodium hydrogen phosphate, 0.02 M sodium hydrogen phosphite, 0.005 M sodium barbital or 0.01 M THAM. The buffers were used in isotonic solution at pH 7.6. Neither phosphate, phosphite nor THAM was toxic at the concentrations tested. The expected toxicity of veronal was demonstrated by failure of the cells to respire in its presence.

TABLE 2

Effect of various buffers on respiration of strain HeLa[1] *in ChS-10, BSS-90*

BUFFER	MOLARITY	OXYGEN UPTAKE, µl hours of incubation 24	53	78
Control		212	475	751
Phosphate	0.02	232	540	783
Phosphite	0.02	235	550	800
Tris (hydroxymethyl) aminomethane	0.01	225	535	810
Veronal	0.005	30	33	41

[1] 840,000 HeLa cells per flask.

DISCUSSION

Oxygen consumption or other measures of metabolic activity have not been accepted commonly (Willmer, '54) as measures of cellular multiplication since many substances may apparently affect respiration without related influence on growth. It has not been appreciated equally that such criticism need not apply to evaluation of the rate of change of oxygen consumption. In this laboratory, the manometric method has already proved a versatile tool for the investigation of cellular physiology and growth. Measurements of the respiration of various populations of HeLa cells demonstrated the correlation of oxygen consumption with cell number. After correction for the reduction in number of viable cells after trypsinization and dispensing (41 to 53% survival), the rate of cellular multiplication was indicated by the change in the rate of oxygen consumption. The rate of multiplication decreased with increasing cell inoculum until a maximum population was attained. For application of manometry to nutritional studies, it is therefore necessary that the initial population be sufficiently small to permit adequate multiplication. This adjustment is particularly important in the evalution of maintenance media.

Maximal rates of respiration were obtained in systems containing human serum, 40% by volume. Greater concentrations of human serum did not enhance the respiration; lesser concentrations gave reduced rates. Added glucose served to sustain the metabolic rate in systems containing low concentrations of serum. Respiration of cells in a 10% serum medium without additional glucose fell off only after 49 hours of incubation when large cell populations were employed. The decrease was particularly marked after 72 hours. The rapid decreases in respiration with low concentrations of serum suggests the exhaustion of an essential nutrient(s) in serum.

For short-term maintenance of a constant rate of respiration, balanced salt solution containing 10% serum was adequate. Respiratory rate was best maintained in well buffered systems; for this reason, a modified maintenance solution was employed frequently. A slight acceleration of respiration in the presence of 20% chicken serum and a negative acceleration in the absence of serum suggested that the maintaining capacity of 10% ChS medium reflected a balance between cellular multiplication and death. Human serum was a better source of limiting factor(s) than was chicken serum in equal concentration. The nature of the limiting factor(s) is currently under investigation in this laboratory. For maintenance of respiration, the complex MS solution with 10% serum was not found superior to the simpler balanced salt solution containing 10% serum. The demonstrated inadequacy of Parker's synthetic medium 703 for maintenance of HeLa cell respiration agrees with an observation by Scherer (personal communication) that strain HeLa cells failed to maintain or grow in this medium. The observation that cells will respire in balanced salt solution for 24 hours is evidence for an endogenous supply of nutrients and energy.

Yeast extract, 0.1%, significantly stimulated the respiration of HeLa cells. A serum-sparing effect was apparent: the rate of respiration of cells in 10% human serum and 0.1% yeast extract was essentially the same as that of cells in 40% human serum without yeast extract. Since human serum in excess of

40% did not stimulate increased respiration, it is suggested that the limiting factor in human serum differs from that in yeast extract.

The lack of toxic effect on HeLa cells of the phosphite, phosphate and THAM solutions indicated that these materials might be used to increase the buffer capacity of the medium.

SUMMARY

The manometric method has been applied in these studies for: (a) estimation of cellular growth, and (b) evaluation of some complex media and buffers for use on HeLa cell cultures. Measurements of respiration of different populations of strain HeLa cells demonstrated that oxygen consumption was correlated with cell number. Cellular multiplication was indicated by the rate of change of oxygen consumption rate. The multiplication rate was inversely proportional to inoculum size; the respiration rate of large initial populations remained almost constant with time. Trypsinization and dispensation procedures destroyed from 47 to 59% of cells.

The proportion of human serum (40% by volume) incorporated commonly in media for cultivation of HeLa cells was proved optimum. The amount of glucose commonly added to media in balanced salt solution limited respiration after 49 hours of incubation, when large cell populations were employed. Cells maintained a steady rate of respiration for at least 4 days when bathed with ChS-10, H-90 medium. Media containing ChS-20, H-80 promoted a slight acceleration of respiration, while H-100 discouraged respiration. The complex MS solution and Parker's synthetic medium 703 were not found superior to Hanks's solution for use in maintenance media.

Addition of yeast extract, 0.1%, significantly increased the respiration of HeLa cells. Cells in HuS-10, BSS-90 containing 0.1% yeast extract respired at a rate equivalent to other cells in HuS-40, BSS-60 lacking yeast extract.

Phosphate 0.02 M, phosphite 0.02 M and tris (hydroxymethyl) amino methane 0.01 M buffers were not toxic for

HeLa cells; 0.005 M sodium barbital inhibited respiration completely.

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ISOLATION OF CORTICAL MATERIAL FROM SEA URCHIN EGGS BY CENTRIFUGATION [1]

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EIGHT FIGURES

INTRODUCTION

Although the surface layers of the sea urchin egg are the site of dramatic changes at fertilization, little is actually known about the submicroscopic structure of the surface layers, their chemistry, or the real nature of the physiological changes that are collectively referred to as the *cortical reaction.*

Excluding the jelly, the surface layers of the unfertilized egg that have been recognized are (1) the vitelline membrane or its precursor, (2) the ''luminous hyaline layer,'' which changes color at fertilization when viewed under dark-ground illumination (Runnström, '23), and (3) the ''Harvey-Moser cortex'' containing granules which ordinarily do not move under centrifugal force (E. N. Harvey, '11; Moser, '39). The plasma-membrane has not been directly observed and identified as a structure; however, recent evidence from permeability studies has tentatively localized this structure beneath the cortex (Parpart and Laris, '54). A similar conclusion has been reached on the basis of observations on eggs deprived of strips of cortical material; such eggs did not undergo cytolysis or lose any cytoplasm through rupture of the true cell surface (Allen, '55).

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Two methods have been reported for the isolation of cortical material. Broken hulls of cortex have been recovered after the centrifugation of homogenates of acid-fixed eggs (Motomura, '54). Strips of cortex were isolated by pulling away eggs adhering to glass; these strips responded to insemination (Allen, '55).

The recent realization that the cortex of the sea urchin egg might be an "extra-cellular" structure suggested the possibility that a specific gravity difference between cortex and the rest of the egg might render isolation of cortical material possible by centrifugation without rupture of the cell itself. Such a prospect would be attractive, for cortical material isolated in this way would be free from the criticism of impurity due to physical contamination or adsorption of endoplasmic substances.

Although the possibilities for chemical investigation of isolated cortical material are considerable, the present paper will be concerned only with details of methodology for cortical isolation, and with the structure of the isolated cortical layers.

MATERIAL AND METHODS

Eggs of the sea urchin *Strongylocentrotus purpuratus* were obtained from animals collected along the California coast, during late January and early February, 1956. Animals were kept in moist sacks in a 4°C. cold room in Berkeley, where their gametes remained fertilizable for from 4 to 5 days.

Most experiments were performed on jelly-free eggs which had been treated with acid sea water (pH 4.5) for two minutes. Jelly removal would be an important step toward ultimate chemical analysis of the isolated cortical material, for the jelly is sufficiently heavy to contaminate the bottom fractions.

Separation of whole, jelly-free eggs into halves and quarters was accomplished by layering the eggs over isotonic sucrose (0.96 molar) in plastic centrifuge tubes. These were tilted several times to form a broad gradient between the sea water and sucrose. Separation of light and heavy halves was obtained at 9,000 × gravity for 10 minutes. Eggs subjected to 11,500 × gravity yielded quarters in the same amount of time (fig. 1).

Isolation of cortical material from whole eggs, halves, or quarters was carried out either in the no. 40 angle-head, or in the new SW-39-L "swing-out bucket" rotor of the Spinco Preparative Ultracentrifuge. The SW-39-L rotor was superior in the resolution of layers obtainable. It receives tubes 1.27 cm in diameter and 5.4 cm long. The bottom centimeter was filled with hypertonic sucrose (40–50%), over which was placed 0.96 molar sucrose (approximately isotonic). Eggs in sea water were layered on the sucrose from approximately half way up the tube to within a few millimeters of the top. The tubes were tilted several times to establish a broad density gradient. In some experiments the bottom layer of sucrose was omitted.

Low temperature facilitated fragmentation of the eggs by centrifugal force. Therefore, separation of halves and quarters (E. B. Harvey, '32) was performed in the cold. For the isolation of cortical material, however, it was desirable to centrifuge at room temperature in order that the cortex would be removed before the eggs had become excessively fragmented.

RESULTS

The cortical layer in stratified eggs

The cortical granules of *S. purpuratus* are irregular in shape and vary in maximum diameter from 0.6 to 1.2 μ (cf. fig. 2). The average distribution of granules is about 47 ± 5 per 100 μ^2. In the centrifuged eggs in which these estimates were made (from photographs similar to fig. 2), it is possible that the distribution measured might have been too low, for the force required to stratify the cytoplasmic granules sufficiently to observe the cortex well (10,000 × gravity for 10 minutes) caused a slight centrifugal migration of the cortical granules. This became especially noticeable in the cortical layers of clear quarters, where it was clearly seen that forces of 13,000 to 17,000 × gravity for 15 minutes had somewhat depleted the cortex of granules on the centripetal pole. Since the heavier quarters and the centrifugal poles of the lighter quarters showed a denser distribution of granules, it is probable that at least some of the granule movement was intra-cortical.

Occasionally, however, large granules, unmistakably of cortical origin, were observed in the clear hyaline zone of the endoplasm following centrifugation at forces around 15,000 × gravity. These granules were found to be the heaviest inclusions in the cell (see below). The fact that they did not fall rapidly and more or less freely through the endoplasm indicated that they might have been attached to the centripetal cortex, suspended from a stretched membrane.

Separation of cortical material from whole eggs

Jelly-free whole eggs were layered over 0.96 molar sucrose and the centrifuge tubes tilted to mix the boundaries into density gradients. Sets of two tubes were centrifuged for from 15 to 45 minutes at speeds from 15,000 to 39,000 revolutions per minute. Since halves, quarters and smaller fragments of eggs separated into distinct layers in the density gradient, each was subjected to a different force, depending upon the angular velocity of the rotor and the radius of rotation. The minimum speed at which cortical separation was detected was 20,000 r.p.m. A few egg fragments exposed at this speed to forces of about 21,000 × gravity lost patches of cortical material, and some isolated cortical granules were recovered throughout the medium and at the bottom of the tube. When eggs were exposed to 40,000 × gravity and above, eggs underwent fragmentation into halves, quarters and then into smaller fragments. If the force was applied slowly, the fragmentation probably took place before cortical separation. If the force was applied rapidly, it was evident that the cortex was stripped from many eggs before much fragmentation had occurred; this was clear from the size of the cortical hulls described below (cf. fig. 3). Egg fragments that had been exposed to 60,000 × gravity and above had lost all cortical material. The cytoplasm of these fragments had the same appearance as that of the untreated unfertilized egg. However, several attempts at fertilizing them failed to produce any visible indications that sperm had penetrated, or that the fragments themselves had reacted to the presence of sperm. Most of the fragments were of the order of size of eighths or sixteenths of unfertilized eggs.

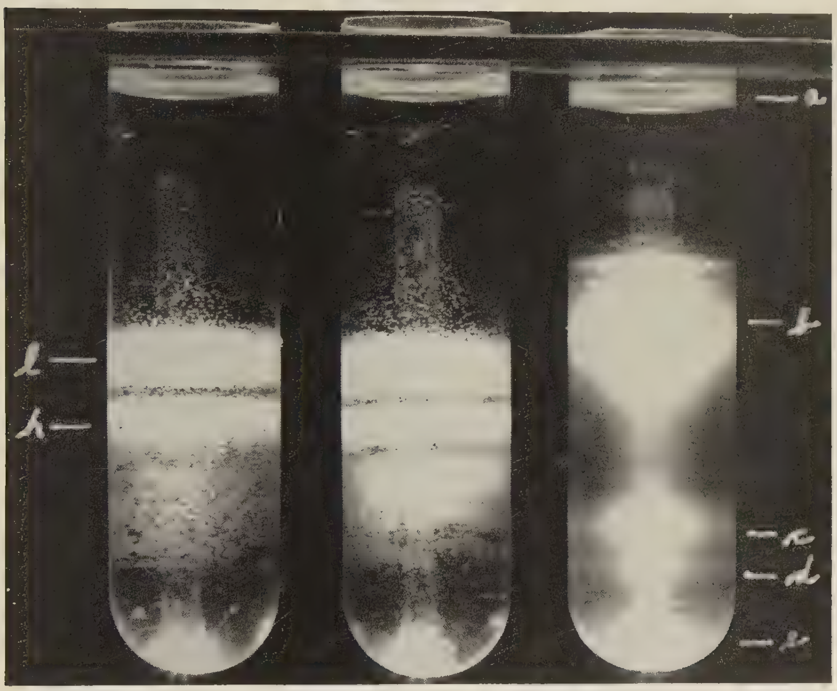

Fig. 1 Plastic centrifuge tubes containing centrifuged sea urchin eggs layered over isotonic sucrose. Left: (l) light and (h) heavy halves. Middle: quarters (lighter two not well resolved). Right: after 45 minutes centrifugation at 35,000 RPM over a double gradient (see text): (a) fragments containing fat-cap material, (b) light fragments, (c) heavy fragments, (d) isolated cortical granules and sheets, (e) thick ''membranes'' and cortical hulls.

Isolated cortical material

1. *Empty cortical hulls,* almost always with granules densely packed on the centrifugal hemisphere but sparse on

the centripetal pole, were usually not plentiful, but of sufficient size to indicate origin from half or quarter egg fragments (fig. 3).

2. Isolated "membranes," too thick to be composed of vitelline membrane material alone, were observed to contain very small dark granules not large enough to be intact cortical granules (compare figs. 4, 5). It seems likely that the "membranes" consisted of the vitelline membranes (or their precursors), plus "luminous hyaline layers" (cf. Allen and Hagström, '55), to which the cortical granules might once have been attached. The small granules attached to these thick

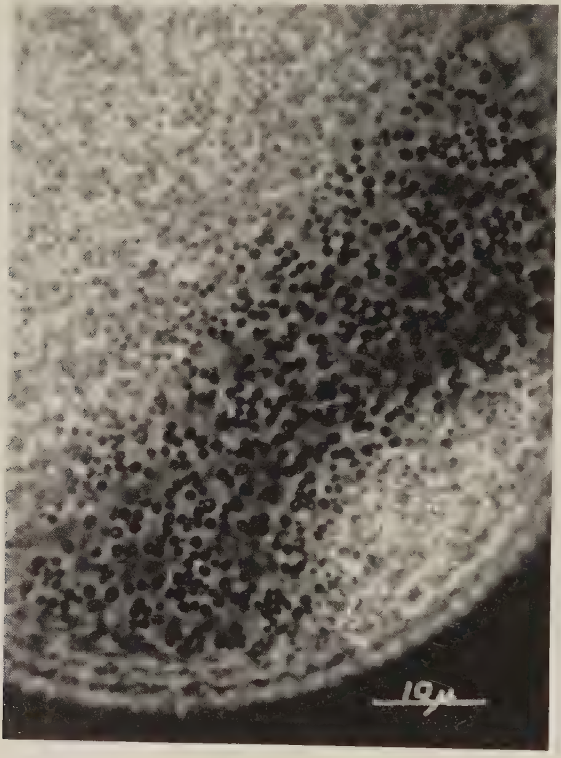

Fig. 2 *S. purpuratus* egg stratified by 10 minutes centrifugation at 10,000 × gravity to show granules in the cortical layer. *Zeiss* phase contrast objective, 100 ×.

"membranes" were probably fragments of cortical granules, for, if they existed as small granules in the cortex, they should have been observed there in the intact centrifuged egg.

3. *Sheets of cortical material,* consisting of cortical gel containing cortical granules, were very common in the bottom sediment following centrifugation. The "membranes" (above), being heavier than the cortical material beneath, evidently became torn away from this material, leaving it isolated from its outer layer (fig. 6). Some cortical strips exhibited fibrils extending in all directions in the plane of the layer; this observation, however, was not common.

4. *Isolated cortical granules* were found distributed throughout the sucrose medium after brief centrifugation at forces above 21,000 × gravity, but they sedimented only upon prolonged centrifugation. It is likely that most of these had been torn loose from the sheets of cortical material. Isolated cortical granules often adhered closely to ane another, showing that they carried around them little or no cortical gel; the cortical granules in cortical sheets were often more crowded than in the cortex of the normal unfertilized egg, but still were separated by discernable spaces (compare figs. 6, 7).

5. *Heavy egg fragments* were found along with cortical sheets and isolated granules in the bottom fractions; they contained mostly cortical material with very little endoplasm and assumed a variety of shapes, the most common being spheres and cylinders. Upon dialysis against sea water, many of the fragments at the bottom disintegrated; of those which remained intact, many responded to the presence of spermatozoa by disintegration. Sometimes two heavy fragments were connected by long fibers which evidently consisted of extended cortical material (fig. 8).

6. *Droplets of high refractive index* were often seen in the bottom fraction. A few times these granules were observed to form from cortical gel undergoing cytolytic changes resulting from excessive heat from the illumination source (fig. 7).

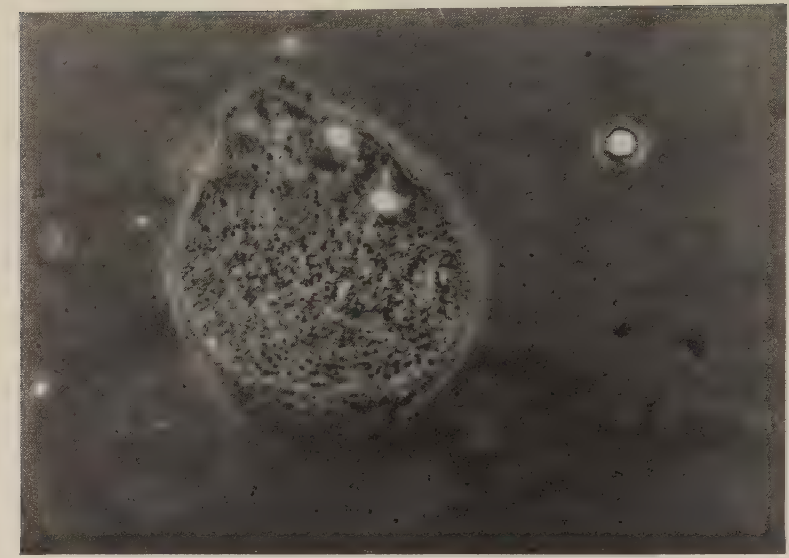

Fig. 3 A hull of cortical material. Note depletion of granules at centripetal pole. Centrifugal force 35,000 × gravity for 15 minutes.

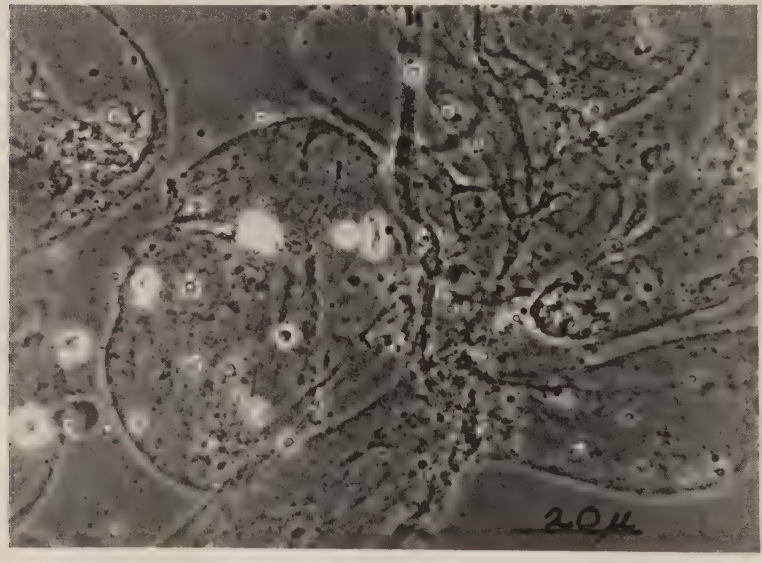

Fig. 4 Isolated ‘‘membranes’’ (see text). Obtained as in figure 4.

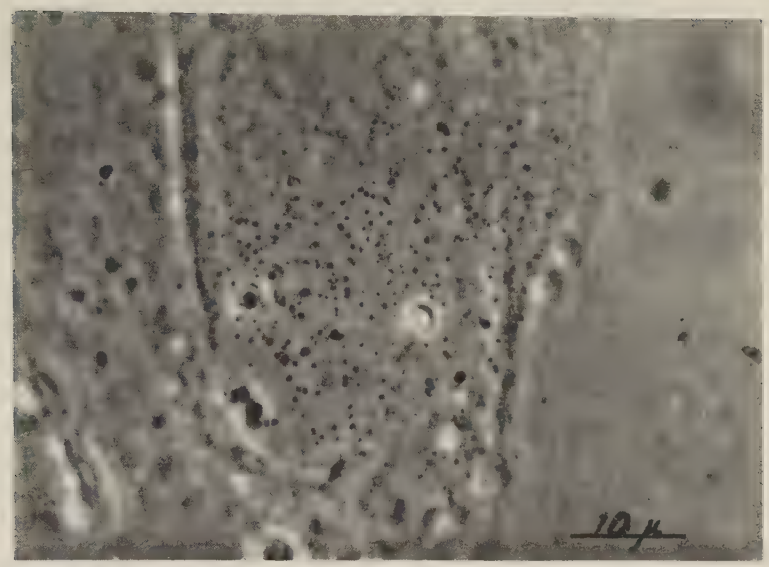

Fig. 5 Small granules believed to be cortical granule fragments attached to the isolated "membranes" (see text).

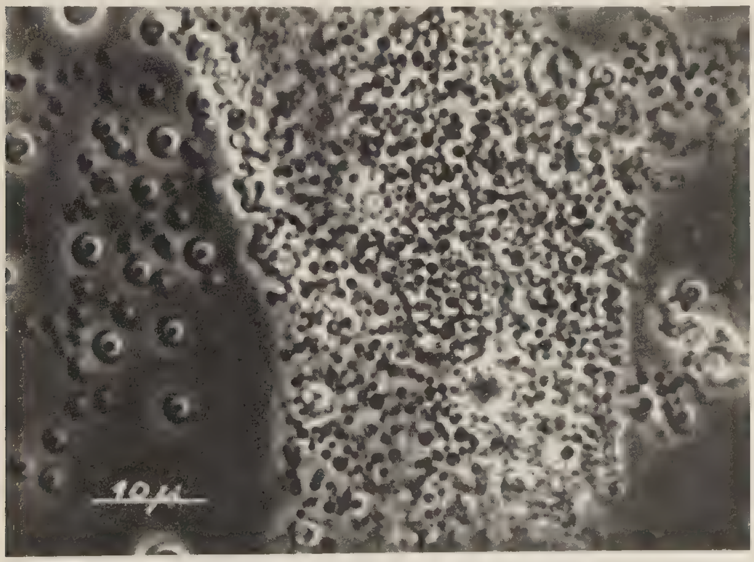

Fig. 6 A sheet of isolated cortical gel containing cortical granules probably somewhat concentrated by the effects of centrifugal force.

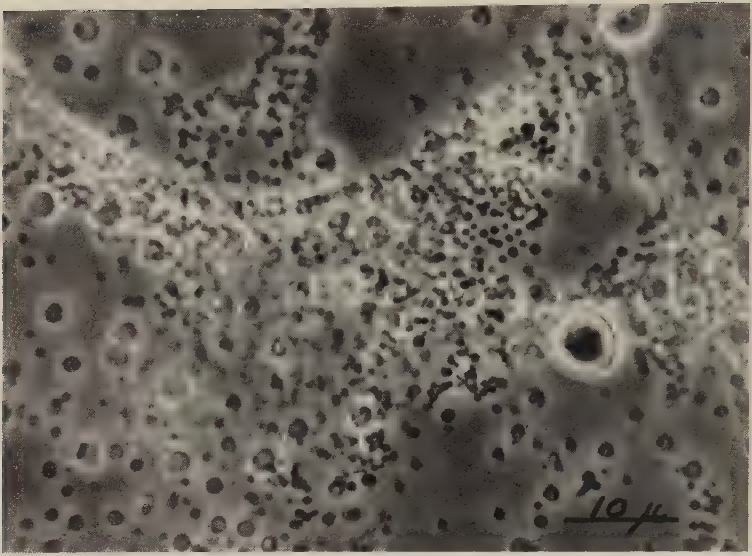

Fig. 7 Sheets of cortical material in the process of disintegrating; note the highly refractile droplets which form apparently as a result of cortical disintegration.

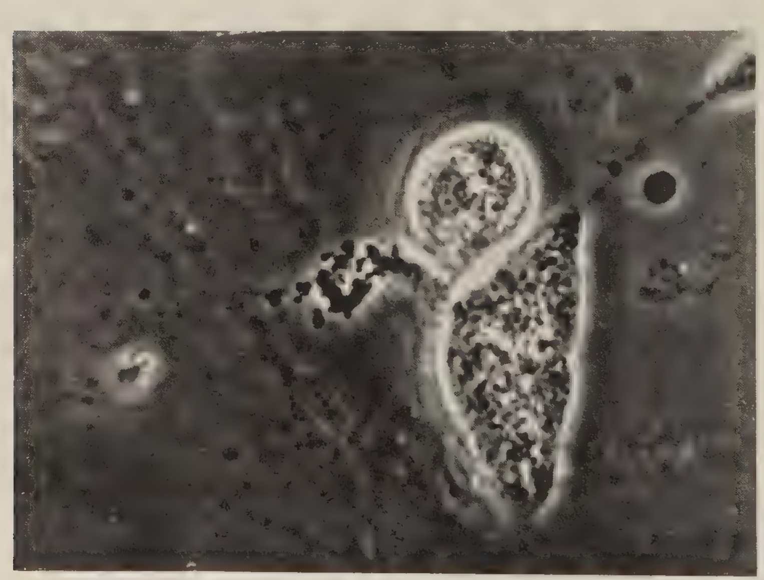

Fig. 8 Heavy egg fragments consisting mostly of cortical material with a small amount of yolky endoplasm. Note the string of cortical material attached to the larger fragment.

Separation of cortical material from egg halves and quarters

In the process of separation of cortical material from whole eggs, one of the first effects of centrifugation is to fragment the eggs into halves and quarters. Accordingly, it was not surprising that the yield of isolated cortical material did not differ qualitatively when cortical isolation was carried out on each of the halves and quarters of the egg. The results were different, however, with respect to the relative quantities of the different kinds of cortical material obtained. Fewer and smaller cortical hulls were obtained from centrifugation of light quarters, and heavy fragments were almost entirely lacking. It might be concluded that the purest preparations of isolated cortical material would be obtained from the light quarters alone. This procedure has the disadvantage of decreasing the yield, however, and if slight impurities consisting chiefly of yolky cytoplasm could be tolerated, it would be preferable to begin with whole eggs or light halves.

Determination of the specific gravity of cortical material

In a double density gradient (sea water over 0.96 molar sucrose, over 1.17 to 1.46 molar sucrose) it was possible to estimate the specific gravity of some of the cortical components. Isolated granules sank in concentrations of sucrose less than 1.32 molar, but remained suspended in more concentrated solutions. The specific gravity of 1.32 molar sucrose at 20° is approximately 1.168, but since the granules were somewhat shrunken in this hypertonic environment, it is probable that their specific gravity was somewhat less. Sheets of cortical material remained with the isolated granules, indicating that the cortical gel was not very different from the granules in specific gravity. Isolated ''membranes'' sank even in 1.75 molar sucrose, the most concentrated solution used. No concerted attempt was made to obtain high resolution between layers of cortical material.

The stability of isolated cortical material in sucrose and in sea water

Isolated cortical material kept its structural integrity for several hours in sucrose solutions. Upon dialysis against sea water, many of the strips or hulls of cortical material became slightly "brownish." Several attempts to fertilize dialyzed cortical material did not produce evidence of response to insemination. However, it could not be proven that none of the material had responded. No attempts were made to facilitate a response to insemination by the addition of favoring substances.

DISCUSSION

The removal of the cortex without dispersing the cell contents constitutes very strong evidence for the presence of a subcortical membrane as postulated by Parpart and Laris ('54) and by Allen ('55). The presence of such a sub-cortical membrane might have significance in determining the mechanism of the propagation of the cortical reaction, especially as this membrane appears to be the plasma-membrane (Parpart and Laris, '54). Alterations of the permeability of this membrane might influence not only cortical conduction, but also cortico-endoplasmic interchange after the cortical reaction, when the "fertilized cortex" apparently "induces" the migration of pigment granules to a subcortical location (Allen and Rowe, '55).

It is interesting to note that E. B. Harvey ('34) first observed that the hyaline layer, which is a major part of the cortex of the fertilized egg, was stripped from the surface of the sea urchin egg by moderate centrifugal forces. The present writer, in unpublished experiments with *Arbacia* eggs, has found that pretreatment of unfertilized eggs with chymotrypsin (0.10% for 15 minutes) permits subsequent isolation by centrifugation of hyaline layers in considerable quantity.

The structure of isolated cortical material may provide clues to the structure of the surface layers of the intact egg.

In a previous report on the isolation of cortex by stripping eggs attached to glass (Allen, '55), it appeared likely that the entire surface of the egg was removed and isolated (i.e. the cortical gel layer with its granules, the luminous hyaline layer and the vitelline membrane or its precursor). In the present experiments, all of these components seem to have remained together only in the cortical hulls, which were the least common of the forms of isolated cortical material. The isolated "membranes," which constituted the heaviest fraction of cortical material, probably became torn away from the inner cortical gel layer through a difference of specific gravity. These "membranes" almost certainly contained more than the vitelline membrane or its precursor, for the vitelline membrane of the unfertilized egg is extremely thin; in fact, there is some doubt that it exists as a membrane on the egg surface prior to fertilization (cf. Allen and Hangström, '55). The thickness of the isolated "membranes" and the fact that they contained small granules indicated that another inner layer probably accompanied the vitelline membrane. This must have been the "luminous hyaline layer," and the small granules must have been fragments torn from cortical granules attached in this layer. Such an attachment would partly explain (1) the resistance of cortical granules to stratification under centrifugal force, and (2) the suspension of cortical granules which were pulled into the hyaline endoplasmic zone of the unfertilized centrifuged egg.

The cortical gel layer, which was isolated in sheets or strips of material, must possess considerable rigidity of its own to withstand the shearing forces that must have been imposed by the tearing away of its outer layers. The observation of fibrils extending from strips of cortical gel might indicate a structural basis for the observed cortical rigidity. The failure of fibrils to appear in all preparations of isolated cortex might mean either that (1) the fibrils themselves were artifacts produced by sucrose, centrifugal force or a state of isolation from the rest of the cell, or (2) that the fibrils had been destroyed in most cases by one of the above treatments.

Although it is not possible to decide definitely which of these possibilities is correct, the latter seems more probable, for it was observed that strips of cortex isolated by pulling the egg away from adhering glass surfaces nearly all possessed such fibrils. Moreover, the cortex isolated in this manner was capable of response to insemination (Allen, '55). The phenomenon of the formation of highly refractive droplets from disintegrating cortical gel is interesting and possibly important, but its mechanism and significance are not entirely clear.

The possibilities of the method described in this paper for the investigation of the chemistry of the cortex seem preferable to those offered by the method of Motomura ('54). The present writer hopes to return to this problem at the earliest opportunity. It is unusually good fortune when cellular structure provides the possibility of isolating a component (even though "extracellular") without serious danger of impurity through direct physical contamination or adsorption of enzymes or other substances from the ground substance of the cell. The present method at least promises that what is recovered in the sediment will be cortical material, although the fact that this material does not appear to be physiologically active may indicate that something has been lost on isolation.

This study indirectly points to the importance of the cortex for fertilization. As Chambers ('21) showed by microdissection studies, egg fragments without cortical material do not respond to insemination.

Interesting as they may be, studies on the kinetics of the cortical change alone, or of the physiological effects of the cortical change will not elucidate its mechanism in molecular terms. Direct chemical investigations of the egg cortex are required before the mechanism of the cortical reaction can be properly elucidated.

SUMMARY

A method is described for the isolation of cortical material from the egg of the sea urchin, *Strongylocentrotus purpuratus*,

by centrifugation. Separation of cortical material from the rest of the egg was made possible by a large specific gravity difference between the lighter egg fragments and the heavy cortical layer. This was accomplished without disruption of the cell fragments and without dispersion of the endoplasm of the cells from which the cortical material was removed.

Isolated cortical material in the form of (1) empty hulls of cortical material, (2) isolated ''membranes,'' actually consisting of the vitelline membrane or its precursor plus the luminous hyaline layer, (3) sheets or strips of cortical gel layer (torn away from the outer membranous layers), (4) isolated single cortical granules devoid of cortical gel material, and (5) heavy egg fragments consisting almost entirely of cortical material. These fragments were the only ones containing any endoplasmic material, and they could be eliminated by selecting only the light quarters for subsequent isolation of cortical material.

Egg fragments deprived of cortical material did not respond to insemination when returned to sea water; isolated cortical material for the most part failed to respond as far as could be determined. However, the heavy fragments described above were occasionally seen to disintegrate upon return to sea water or in the presence of spermatozoa.

The possible significance of isolated cortical material is discussed in relation to the structure of the surface layers of the egg and the mechanism of the cortical reaction.

I am very much indebted to Dr. Daniel Mazia for the pleasure and privilege of performing this work in his laboratory in the Department of Zoology of the University of California, Berkeley.

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CELL DIVISION:
A PRESSURE-TEMPERATURE ANALYSIS OF THE EFFECTS OF SULFHYDRYL REAGENTS ON THE CORTICAL PLASMAGEL STRUCTURE AND FURROWING STRENGTH OF DIVIDING EGGS (ARBACIA AND CHAETOPTERUS)[1]

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NINE FIGURES

INTRODUCTION

Functional significance of protoplasmic gelations

Rapid reversible gelations have been observed in the protoplasm of various cells for many years. However, the significance of these reactions in relation to the specific activities of the cell was not appreciated until about 1925. Then the work of Mast ('26), Lewis ('39), Marsland ('38, '48 and '50) and Pease ('40) began to indicate that gelation reactions are instrumental in the performance of mechanical work in many kinds of cells. The evidence suggests that gelated parts of the cytoplasm develop an inherent contractility. Thus cytoplasmic gelations seem to be involved in the execution of a variety of cellular movements, namely: (1) amoeboid movement (Mast, '26; Marsland and Brown, '36; and Landau,

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Zimmerman and Marsland, '54); (2) the anaphase movements of chromosomes (Pease, '41); (3) protoplasmic streaming in plant cells (Marsland, '39c); (4) the migration of pigment granules in unicellular pigmentary effectors (Marsland, '44); and (5) the furrowing process in dividing egg-cells (Marsland, '39a and b, and '48; and Landau, Marsland and Zimmerman, '55).

It seems almost self-evident that the development of contractility in an essential fluid system such as protoplasm would presuppose the formation of some kind of gel structure. Assuming that the contractile force originates from the folding of elongate protein molecules or molecular aggregates (cf. Goldacre and Lorch, '50; Goldacre, '52), it is difficult to understand how such a folding would be effective in performing work unless the extended protein units were interlinked, forming a continuous and fairly extensive system in the cytoplasm. In fact it is generally agreed (cf. Ferry, '48; and Kopac, '50) that gelation represents the formation of a three-dimensional network of fibrillar units present in the system. Thus the contraction of a gel structure would represent a rapid kind of syneresis, whereby the protein components of the colloidal network undergo a forcible folding without relinquishing the intermolecular linkages. Subsequent to folding, moreover, when the extended units have assumed a more globular form, the system would revert to the sol condition, merely by the loosening of the intermolecular bonds.

Nature of protoplasmic gelations

It is important to realize that protoplasmic gels, as compared to more familiar types like gelatin or agar, belong in a distinctly different category. Gelatin gels, according to the criteria of Freundlich ('37), may be taken to represent the Type I system, in which the sol → gel change occurs exothermically and with a definite volume *decrease* ($\Delta V = -185 \text{ cm}^3/\text{mol}$; Marsland and Brown, '42). In the gelatin system, therefore, gelation is inhibited by increasing temperature, whereas it is favored by increasing pressure (Marsland and Brown, '42). Protoplasmic gels, on the other hand, display the opposite behavior. They are like methyl cellulose gels, which may be classified as Type II systems. In such systems, the sol → gel reaction represents an endothermic process with a concomitant volume *increase* ($\Delta V = +110\ \text{cm}^3/\text{mol}$, calculated on the basis of the average protein content). Accordingly, it is not surprising to find that high pressures applied to various living cells produce a drastic weakening of the plasmagel structures, with a marked inhibition of cellular movement. Moreover these effects of high pressure are definitely augmented at lower temperatures (see Marsland, '50; Marsland and Landau, '54; and Landau, Zimmerman and Marsland, '54). Thus pressure and temperature represent a combination of variables which provide a useful method for studying the action of various agents upon the sol ⇌ gel equilibria in the cell and for analyzing the relationship between gelation and physiological activity.

Metabolic energy in relation to gelation

Granting that the building of a protoplasmic gel structure requires metabolic energy which later appears as mechanical work when the gel contracts, an investigation of the available energy sources becomes important. Some early work (Marsland and Brown, '42) indicated that the ATP system might be involved and a number of later reports (Landau, Marsland and Zimmerman, '55; Kriszat, '49 and '50; Runnstrom and Kriszat, '50a and b; Hoffmann-Berling, '54c; Hoffmann-Berling and Weber, '53) have tended to confirm this viewpoint. Consequently the present study of the effects of sulfhydryl inhibitors on the structural state of the cortical plasmagel system and on the furrowing capacity of dividing egg-cells, represents a continuation of this line of work.

Two sulfhydryl inhibitors, namely mersalyl acid (salyrgan) and para-chloromercuribenzoate, were selected because they have been studied by a number of previous investigators. H. H. Weber and co-workers ('55a and b), for example, showed that salyrgan produces a reversible inhibition of contraction in glycerol-extracted fibroblasts and muscle fibers (see also Kuschinsky and Turba, '50a and b, and '51), and Singer and Barron ('44) found that p-chloromercuribenzoate effects a reversible inhibition on the ATP-ase activity of myosin. Moreover, the work of many other investigators such as Hammett ('29 and '34), Voegtlin and Chalkley ('30), Rapkine ('31), Brachet ('50), and Mazia ('54), indicates that the thiol compounds play a very active and important metabolic role, especially during the cleavage divisions and the early growth and developmental processes of the embryo.

Sol-gel reactions in relation to mitosis

The centrifugal studies of Dugald Brown ('34) indicated that fertilization initiates a series of very rapid drastic changes in the gelational state of the cortical cytoplasm of the egg. In the Arbacia egg, at least, the great fortification of the plasmagel structure which accompanies the onset of the first cleavage mitosis appears to be instrumental in the achievement of the furrowing reaction, but the significance of the earlier fluctuations has remained obscure. Brown's experiments gave only a rough approximation of the magnitude of the gelational changes and, since the temperature was not controlled, and exact timing of the changes in relation to the movements of the pronuclei and other important events associated with fertilization could not be determined. Consequently it was decided to re-investigate the situation, using accurately controlled temperature conditions, and using higher centrifugal forces, adequate to provide for a quantitative evaluation of the magnitude of the changes of gel structure.

Summary of objectives

In short, the three main purposes of this investigation were: (1) to obtain more accurate measurements of the magnitude and timing of the sol $\rightleftharpoons$ gel changes which occur in the cortical cytoplasm of the egg-cell from the time of insemination until the completion of second cleavage; (2) to relate these gelational changes to the syngamic and mitotic events which occur concomitantly; and (3) to investigate the energy sources and mechanisms of these gelations by studying the effects of two selected sulfhydryl inhibitors, namely, p-chloromercuribenzoate and mersalyl acid (salyrgan).

MATERIALS AND METHODS

Shedding and insemination

For Arbacia, the eggs were obtained sometimes by the electrical shedding method of E. B. Harvey ('52 and '53) and sometimes by the usual KCl injection method. These two methods gave approximately the same percentages of membrane lifting and cleavage, provided the KCl-treated eggs were washed in two changes of sea water prior to insemination. For insemination, two drops of dilute (1 drop of dry sperm to 15 ml sea water) sperm suspension were added to about 20 ml of egg suspension.

For Chaetopterus, the usual method (Heilbrunn and Wilson, '48) was used for obtaining and handling the gametes. However, Chaetopterus eggs show signs of deterioration quite soon after shedding into sea water, and therefore each successive experiment required a separate shedding. In any one series of experiments, eggs from the same female were always used.

Chemicals

Salyrgan (mersalyl acid) was obtained from Winthrop-Stearns, N. Y., and fresh solutions in sea water were made up daily. At a concentration of 10^{-3} M, the salyrgan solutions displayed a pH of 8.02, which is very close to the normal sea water value (8.03).

The sodium salt of para-chloromercuribenzoic acid was purchased from Sigma Chemical Co., St. Louis, Missouri. This compound proved to be insoluble in normal sea water, and consequently Moore's calcium-magnesium-free sea water was substituted as the immersion medium. The pH value of the p-chloromercuribenzoate solutions (5×10^{-5} M) used in the experiments was 6.7.

Pressure-temperature apparatus

The temperature control chamber, microscope pressure chamber and the pressure pump were the same as those described by Marsland ('50). This apparatus permits the cells to be observed at magnifications up to 600 diameters, while they are subjected to pressures up to 15,000 lbs./in.^{2}, at selected temperatures between 5° and 40°C., with a maximum temperature variation of ± 0.3°C.

Centrifugation

Measurements of the consistency (gel strength) of the cortical plasmagel layer as a function of both temperature and pressure were made using a pressure-centrifuge head similar to the one described by Brown ('34). A needle valve permits a "sealing-in" of the pressure on one side of this head, while the other side contains control specimens at atmospheric pressure. This set-up enables the control and pressure-treated eggs to be subjected simultaneously to equal centrifugal forces over a wide range. Thus with various belt-pulley ratios up to 3.38 : 1 and with a centrifugal radius of 6.8 cm, forces as high as 68,000 × G could be obtained from a 0.5 h.p. Dumore motor driven at speeds up to 8,900 rpm. In most of the experiments, however, the centrifugal force was standardized at 41,000 × G, although 27,800 × G was used in some of the preliminary work. The procedure was very carefully standardized so that the centrifugation was started exactly one minute after the pressure was built up in the centrifuge head.

A 1–2 mm cushion of 0.95 molal sucrose was placed on the bottom of both the pressurized and control centrifuge chambers. This prevented the eggs from being packed too tightly at the bottom.

In order to maintain a uniform centrifugal force throughout the experiments, the centrifuge speed was checked stroboscopically at regular intervals by means of a "Strobotac." Voltage adjustments were made with a variable transformer (Variac) powered from the 115 volt line. A voltmeter was placed between the Variac and the centrifuge motor which permitted a continuous reading of the voltage. Thus fluctuations in the power line during centrifugations could be eliminated manually by means of the variable transformer.

Temperature equilibration

Proper temperature equilibration was assured by keeping the pressure apparatus, the centrifuge and all glassware and solutions inside of the temperature-control chamber for an adequate time prior to usage.

For experiments at 20° and 25°C., all stock and experimental eggs were kept at the experimental temperature while the day's work was in progress. However, at 30°C., in order to prevent deterioration, the stock batch was kept at room temperature. Then for each experiment, 5 drops of concentrated egg-suspension were added to 20 cm^3 of sea water at 30°C. just prior to insemination, and this sample was maintained at 30° during the experiment. At 15°C., in order to avoid a prolonged delay in the appearance of furrows, the eggs were inseminated at room temperature and allowed to develop at this temperature for 30 minutes. Then three drops of fertilized egg-suspension at room temperature was added to 20 cm^3 of 15° sea water in the lucite vessel and this was placed in the pressure chamber which contained 80 cm^3 of sea water already equilibrated at 15°C. Thus the eggs remained at 15°C. for at least 25 minutes before the furrows appeared and the furrowing strength was tested.

During all centrifugations the refrigerating unit was operating so that the heat generated by the rotation of the motor and the head did not produce any significant elevation of the temperature in the control housing.

THE EXPERIMENTS

Cortical sol-gel changes following insemination

Insemination to first cleavage (Arbacia). These experiments followed the method of Brown ('34), except that: (1) the temperature was controlled at 20°C.; (2) the measurements were made at shorter (one minute) intervals; (3) the centrifugal force (41,000 × G) was higher; and (4) the centrifugation pressure (8000 lbs./in.^{2}) was slightly greater. All of these factors tended toward a greater refinement of the measurements. The temperature control and frequency of measurements gave a more precise timing of the sol $\rightleftharpoons$ gel fluctuations and the higher force and pressure, which allowed all the eggs to reach a standard end-point of centrifugal displacement, gave a more accurate evaluation of the gelational states.

Following insemination (see fig. 1) the strength of the gel structure of the cortical layer of cytoplasm of the egg — as judged by the resistance it offers to the centrifugal displacement of the pigment bodies (red chromatophores) embedded in it — rises very sharply to a new order of magnitude. In fact the stiffness of the cortical gel becomes so great 12–14 minutes after insemination that it requires more than 8 minutes of centrifuging even at the very high force of 41,000 × G to achieve a standard degree of displacement of the pigment bodies from the centripetal half of the egg, *if the centrifuging is done at atmospheric pressure.* In order to keep the centrifugation periods within reasonable limits, therefore, all the measurements were made at high pressure (8,000 lbs./in.^{2}). This procedure gave an added advantage. Pressures of this magnitude temporarily inhibit the developmental processes, which tended to stabilize the measurements.

In relative terms, the first peak (fig. 1) of plasmagel strength, which is achieved some 13 minutes after insemination, represents more than a 50-fold increase from the value which is obtained in the unfertilized egg. Thus, unfertilized eggs centrifuged for only three seconds at 41,000 × G at

8,000 lbs./in.², display a clearing of all but 3–6 pigment bodies from the centripetal half of the cortex (standard end-point adopted in these experiments) as compared to 160 seconds which is required for eggs centrifuged 13 minutes after insemination.

After the first peak (fig. 1), a second and then a third high point follow quickly in the gel strength curve of the first division cycle.

The second, which reaches a maximum at 23 minutes after insemination, resembles the first in that it is very short-lived and displays about the same magnitude. But the third peak, which achieves a maximum at 33 minutes, reaches a significantly higher level and endures for a much longer period. In fact, the third gel strength peak of the first division cycle persists until the end of the cycle when the eggs have just finished furrowing. Then the cortical gel strength drops very abruptly to a much lower level.

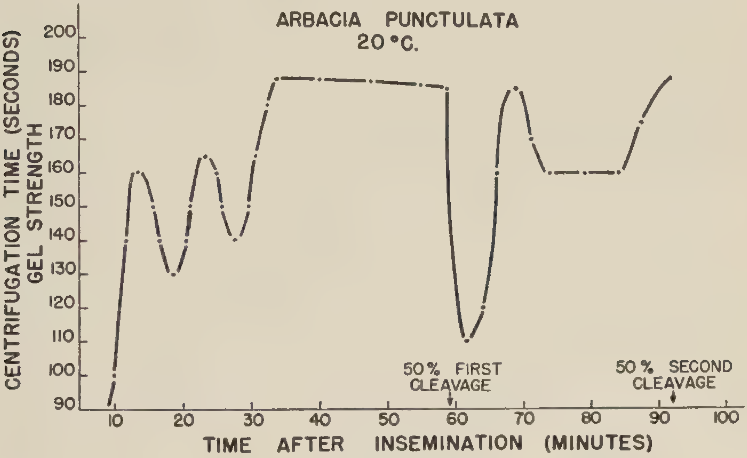

Fig. 1 Cortical sol-gel changes following insemination. Pressure-centrifuge measurements of the structural state of the plasmagel layer of the Arbacia egg made at high pressure (8000 lbs./in.²) and high force (41,000 × G).

The second division cycle. The second division cycle (fig. 1) tends to resemble the first, except that it displays only two gel strength peaks, both approximately of the same height. The first follows quickly after the post-furrowing low and reaches a short-lived maximum 10 minutes after the cleavage time. The second rise, on the other hand, starts about 7 minutes prior to the time when 50% of the eggs display the second cleavage furrow and endures until the furrowing is finished.

The first gel-strength rise. Although it was not difficult to evaluate the very weak gelational state of the *unfertilized* eggs, no measurements could be obtained during the first 8–9 minutes following insemination (see fig. 1). During this period, eggs exposed to centrifugal forces high enough to displace the cortical pigment undergo elongation and breakage so quickly that time for pigment displacement is not afforded. Apparently the fertilization membrane is exceedingly weak during the period immediately following its lifting. Within 10 minutes, however, it grows tough enough to prevent much elongation of the eggs even in the strong centrifugal field which is needed for pigment displacement.

Variations in the cleavage schedule. Even though the temperature of the experiments was carefully controlled at 20 ± 0.3°C., different batches of eggs (and sometimes even the same batch) showed a significant degree of variation in the timing of the first and second division cycles. In the more than 300 experiments performed in this series, the time at which distinct furrows appeared in 50% of the sample (usually about 200 eggs) ranged from 57 to 65 minutes for the first cleavage and 89–95 minutes for the second with the mean values falling at 59 and 92 minutes respectively. In plotting the data of figure 1, accordingly, a standard timing of 59 minutes for the first cleavage, and 92 minutes for the second was adopted. This was obtained by multiplying the time figures by an adjustment factor which was slightly less than 1.0 for ''slow'' batches of eggs, and slightly greater than 1.0 for ''fast'' batches, depending upon the degree of departure from the adopted standard. In all batches, however, three peaks in the gel strength curve appeared in the interval between insemination and first cleavage and two peaks appeared between the first and second cleavages. In other words, there were small variations in the timing, but the intrinsic pattern of the sol $\rightleftharpoons$ gel changes was constant.

Variations in single centrifugal samples. The eggs of any one sample centrifuged before insemination and during the first 15–20 minutes thereafter displayed a remarkable homogeneity as to degree to which the pigment bodies were displaced from the cortex of the light hemisphere. Thus when the duration of the centrifuging was chosen correctly, about 90% of the eggs displayed the end-point configuration, with the small minority distributed quite equally on the "underdone" and "overdone" side of the end-point. Thus it seems that the initial gelational variation among eggs derived from the same female is quite small. With further time, however, the spread became somewhat greater, probably because a complete synchrony of the developmental processes is not maintained. At later times, accordingly, the majority class (eggs at the end-point) dropped off to about 60% except during the 33–59 minute interval, when the high gelational state was maintained — and during the 75–85 minute period, when the weaker gel structure persisted.

Variations in the magnitude of the sol $\rightleftharpoons$ gel shifts. Early in the experiments it became evident that some batches of eggs were "stronger" than others in regard to the peak gelational states achieved during the division cycles. However, it was also noted that egg-batches which displayed exceptionally high peak values also showed correspondingly higher values in the valleys between the peaks; and a corresponding situation was found for exceptionally "weak" batches. Unfortunately, the time during which good eggs were available allowed for only 4 or 5 measurements at each successive minute during the two division cycles, so that a statistical analysis of the variation would not be truly significant. Each of the points shown in figure 1 represents the average of the 4–5 available determinations at the specified time; and the averaged values of the intermediate determinations (not shown on the graph) tend to fall very closely upon the curve as drawn. Also, it should be said, the maximum departure from the specified peak and valley values, except for one experiment, was not greater than plus or minus 10 units.

The post-furrowing gel-strength drop. Some difficulty was experienced in determining the time course of the sudden weakening of the plasmagel structure which occurs when the furrowing of the egg has been, or is being, completed. By the time of first cleavage the eggs of any batch are slightly out of step so that usually there is a spread of 3–4 minutes between the time when the first and last eggs display incipient furrows. Thus there is a mixed population of uncleaved, cleaving and cleaved eggs when the centrifuging is done within a minute or two after 50% of the sample is seen to be furrowing. Fortunately, however, the pressure treatment (8,000 lbs./in.^{2}) which accompanied the centrifugations permitted the cleaved eggs to be distinguished from the others — because pressure of this magnitude causes a reversal of all furrows except those which have completed the cleavage. Thus eggs pressure-centrifuged for 110 seconds starting one minute after the 50% furrowing time show only about 10% of two-celled specimens and *all* of these display a well-displaced pigment, which is characteristic of a weakened gel structure. The one-celled specimens, on the other hand, display a strong gel structure with very little of the pigment displaced from the cortex, except for a small majority (about 12%) which are almost as well-stratified as the two-celled specimens. Then, one and two minutes later, the percentage of weakly gelled two-celled specimens rises to 30 and 50% respectively, with a majority of the remaining one-celled individuals still in a strongly gelled condition. Accordingly it seems safe to conclude that a drastic weakening of the cortical plasmagel structure occurs either at the very end of the furrowing process, or very quickly (within a fraction of a minute) thereafter.

Premature abortive furrowing

This puzzling phenomenon (see fig. 2) frequently appeared in eggs which had been pressure-centrifuged in the second half of the division cycles. It consisted of a vigorous furrowing, always at right angles to the centrifugal axis, starting as early as 29 minutes before the untreated eggs began to display any furrows. Often the premature furrows cut so deeply into the egg contour that cleavage appeared to be complete. Later, however, at the time when normal furrows were appearing in the control eggs, the premature furrows almost always receded and were replaced by new ones, which always came in at right angles to the old, i.e., with the furrow plane parallel to the centrifugal axis.

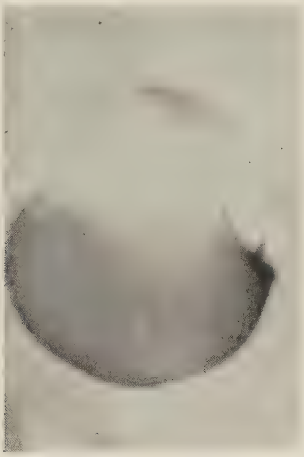

Fig. 2 Premature abortive furrowing (see text).

The premature furrows usually began to appear 2–3 minutes subsequent to the centrifuging. At this time the eggs were virtually spherical. In other words, the slight degree of elongation induced by the centrifugal treatment was, in most cases, not discernible by the time that premature furrowing started.

Premature furrowing appeared, with perhaps rare exception, only in eggs which were at or beyond the centrifugal end-point used in these experiments. This end-point, however, represents a rather drastic displacement of the egg components (see fig. 3). The cortical pigment, mitochondria, and yolk all are thrown well down into the heavy, opaque hemisphere and there is a compact "oil cap" at the centripetal extremity of the light hyaline half of the egg. The nucleus, at least during stages when it can be seen, lies in the hyaline half, very close to the "oil cap" and, presumably, the spindle-aster complex maintains its association with the nuclear material. However, further studies, perhaps utilizing cytological methods, are needed before the conditions which lead to premature furrowing can be determined very precisely.

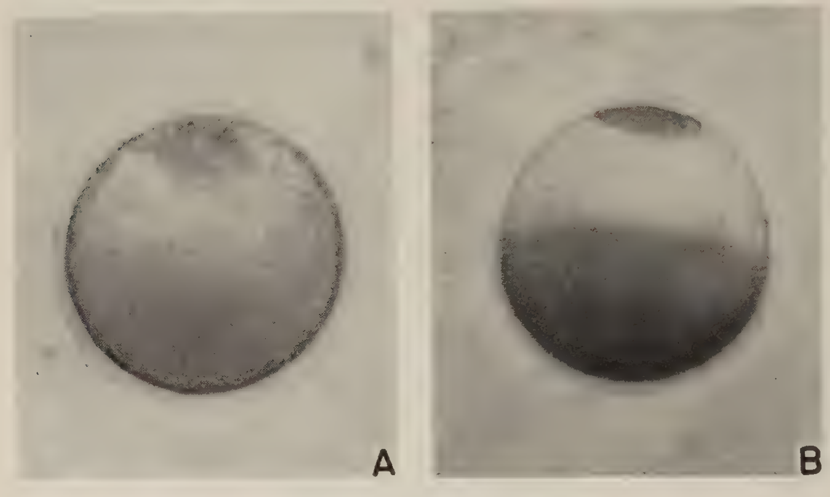

Fig. 3 Photographs showing two eggs *(Arbacia punctulata)* centrifuged simultaneously for 185 seconds at 41,000 × G and 20°C. The control egg (A), centrifuged at atmospheric pressure, displays little, if any, displacement of the pigment bodies of the cytoplasmic cortex. The experimental egg (B), which was centrifuged at high pressure (8000 lbs./in.^{2}), shows a displacement of virtually all of the pigment bodies from the cortex of the centripetal half of the egg. This represents the standard displacement end-point used in the experiments.

In the first division cycle a maximum frequency of premature furrowing occurred about 25 minutes early, with reference to the normal schedule, and in some experiments some 90%

of the eggs displayed the phenomenon. And in the second cycle, there was a maximum frequency at 20 minutes before the normal time. Moreover, premature furrows which appeared not more than 2–3 minutes early sometimes failed to abort. Considerable variation also occurred in the position of the premature furrows. Usually the abortive cleavages were quite equal, but sometimes the furrows were displaced more or less drastically in the direction of the oil cap.

Effects of the sulfhydryl drugs on the sol-gel equilibrium

Concentration, time and other factors. Numerous preliminary experiments, involving the observation of fertilized eggs immersed for varying periods in systematically varied concentrations of the drugs were necessary in order to determine what concentrations and exposure-times were best adapted to the main experiments. The aim was to select a concentration-time treatment that would weaken but not abolish the cleavages. Thus it would be possible to measure the weakening effect by testing the capacity of the eggs to complete cleavage under a variety of pressure-temperature conditions and to correlate these observations with parallel measurements of the gelational state of the cortical cytoplasm.

At higher concentrations, both salyrgan (mersalyl acid) and p-chloromercuribenzoate gave a complete inhibition of cleavage, but at lower concentrations the percentage of eggs capable of going through first and second cleavage became progressively higher. Finally a treatment was chosen which permitted 95–98% of the eggs to cleave, at 20°C.

For *Arbacia,* in the case of salyrgan, the chosen concentration was 2×10^{-3} M (in normal sea water) and the immersion was started 40 minutes after insemination. The treatment delayed the onset of first cleavage by 10–15 minutes, giving a total immersion time (before first cleavage) of 29–34 minutes. Otherwise, however, the eggs seemed to cleave normally except that the percentage of embryos that came through to the blastula stage was reduced to about 60%. At other temperatures the percentage inhibition of cleavage was higher, namely, 40–50% at 30°C. and 70–80% at 15°C.

For *Chaetopterus* at 20°C., an equivalent percentage (95–98) of successful cleavages was obtained using the same concentration (2×10^{-3} M) of salyrgan, when the immersion period was standardized at 17 minutes (counting the two minute delay in the appearance of the polar lobes, which was induced by the treatment). However, the percentage inhibition at higher temperatures was higher, namely about 10 and 50 at 25° and 30°C., respectively.

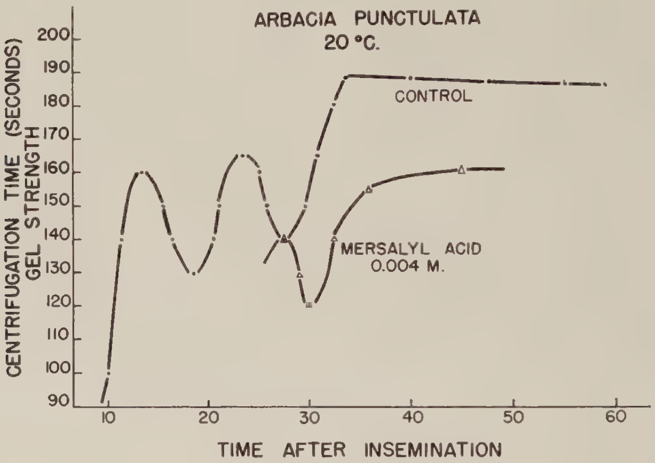

Fig. 4 Salyrgan (mersalyl acid) effect on the sol-gel changes of the first division cycle. Pressure-centrifuge measurements of the gelational state of the cortical cytoplasm of fertilized Arbacia eggs treated with 0.004 M. salyrgan. Both control and drug-treated eggs were subjected to 8000 lbs./in.^{2} pressure at a centrifugal force of 41,000 × G. until the standard end-point was reached.

Only Arbacia eggs were used for the work with p-chloromercuribenzoate. The use of Moore's Ca^{++}-Mg^{++}-free medium for the drug immersions, necessitated using a double set of controls — one in normal sea water and one in the Ca^{++}-Mg^{++}-free medium — since it was found that the absence of the divalent ions had a detrimental influence on both the strength of the furrowing reaction and on the structure of cortical plasmagel. Eggs in Moore's solution showed no delay in the schedule of cleavages. However, few, if any, normal blastulae developed, since the blastomeres tended to dissociate after the second or third cleavage.

The chosen concentration of p-chloromercuribenzoate was 5×10^{-5} M and immersion in the drug was started 30 minutes after insemination. However, prior to placing the eggs in the drug-containing solution, the eggs were washed twice, at 10 minute intervals, by transferring through Moore's solution (0.5 cm^3 dense egg suspension into 9.5 cm^3 of new medium). This treatment allowed for 95–96% cleavage, with only a 1–2 minute delay in the onset of furrowing.

Effects of salyrgan (mersalyl acid) on the sol-gel changes of the first division cycle. These experiments on the Arbacia egg (see fig. 4) followed the same procedures as were used in the other measurements on the structural state of the cortical gel, except that the eggs were immersed in 0.004 M[3] salyrgan sea water solution starting 10 minutes after insemination. The gel strength measurements, which were made at one minute intervals, were not started until the 27th minute, which allowed a minimum immersion period of 17 minutes during which the drug could take effect. Unfortunately, the earlier parts of the gel-strength curve (fig. 4) could not be determined because the eggs could not be fertilized in the drug solution and when the immersions were started sooner than 10 minutes after insemination, cleavage was blocked completely.

As is shown in figure 4, the salyrgan treatment appears to have two main effects. Not only is there a retardation of the gelation reactions, so that the two gel-strength peaks preceding first cleavage are delayed, but also the magnitude of these peaks is reduced significantly. Moreover, the retardation in gel-strength build-ups appears to be correlated with the fact that the onset of cleavage is delayed some 10–15 minutes in the drug-treated eggs; and the weakening effect of the drug on the cortical gel structure seems to be related to the fact that the furrowing strength of these eggs is weakened (see below).

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[3] Some experiments were done using 0.002 M salyrgan, but differences between the normal and the drug-treated eggs were found to be much sharper with the higher concentration.

Pressure measurements on the pre-cleavage cortical gel structure of drug-treated eggs. In these experiments, the gelational state of the cortical cytoplasm of treated and untreated Arbacia eggs was measured as a function of pressure during the pre-cleavage high plateau of the gel-strength curve. In fact, all the measurements were made exactly 10 minutes prior to the time when the control eggs were scheduled to be at the 50% furrowing stage. This timing gave two advantages. First it provided a period during which the gelational state remained at a steady level; and second, it allowed ample time for a critical study of the centrifugal displacement of the cortical pigment bodies before this displacement began to be altered by the onset of furrowing. Moreover, these measurements seemed important in relation to the further study (see below) of the effects of pressure on the furrowing capacity of the drug-treated and untreated eggs.

The salyrgan measurements of the pre-cleavage cortical gel strength as a function of pressure are shown in figure 5. Here it may be noted that the salyrgan treatment consistently weakens the gel structure, but it does not change its susceptibility to solation under increasing pressure. In fact, the control and salyrgan curves display essentially the same slope, but consistently, at each different pressure level, the salyrgan values are about 20% lower than those of the controls.

These salyrgan measurements were made before a higher speed centrifuge became available. Thus the centrifugal force employed was only 27,800 × G and the end-point displacement of the cortical pigment was less complete than that used in the later experiments. This lower force tended to prolong the centrifuging so that measurements at the lower pressure levels, where the gel strength is exceedingly great, were not feasible. However, the measurements in the higher range were very consistent. Each of the points shown on the graph represents the average of not less than three measurements and the variation in the values obtained for any point was, with rare exception, not greater than ± 5%.

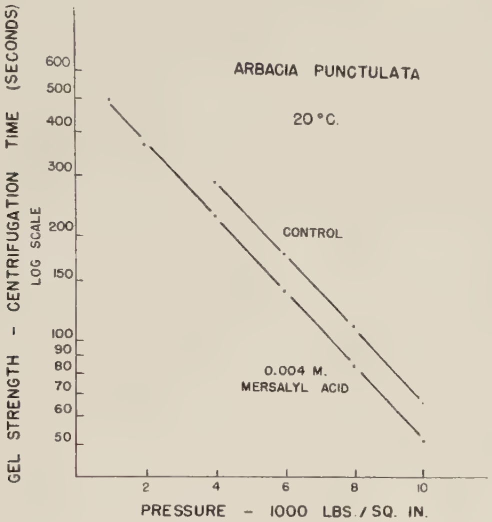

Fig. 5 Effects of salyrgan (mersalyl acid) on the gelational state of the cortical cytoplasm of Arbacia eggs just prior to cleavage. The standard centrifugal force used in these experiments was 27,800 × G and each measurement was started exactly 10 minutes prior to the appearance of the cleavage furrows.

Similar measurements of the pre-cleavage cortical gel strength as a function of pressure, using p-chloromercuribenzoate, another sulfhydryl blocking drug, are shown in figure 6. In these experiments, however, the centrifugal force was higher (41,000 × G) and Moore's calcium-magnesium-free sea water served as the immersion medium for both the control and the drug-treated eggs. These two factors tended to reduce the centrifuging times, but did not change the essential relationships.

In figure 6, again it may be seen that the main effect of the p-chloromercuribenzoate is to weaken the cortical gel structure without changing its susceptibility to pressure solation. To be sure the gel strength level of the controls in the Ca^{++}-Mg^{++}-free medium is rather low, compared to the normal sea water values, but the drug effect, which lowers the gel strength still further by approximately 21% at each different pressure, is not obscured. These measurements also displayed a remarkable consistency. Usually the 3–4 values which were averaged to determine each point did not vary by more than ± 3% and the maximum variation never exceeded ± 7%.

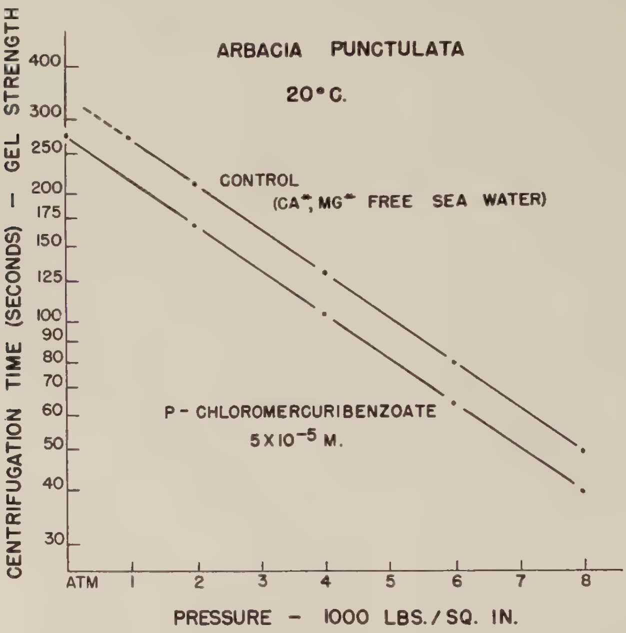

Fig. 6 Effects of p-chloromercuribenzoate on the pressure-gel strength relations of the cortical cytoplasm of the Arbacia egg immediately prior to cleavage. Each centrifugation was started exactly 10 minutes prior to the appearance of the cleavage furrows and a standard force of 41,000 × G was employed.

Effects of the sulfhydryl drugs on the furrowing strength

Previously it has been shown (Marsland, '50; Marsland and Landau, '54) that the minimum pressure required to block the furrowing of different eggs may be used as a measure of the strength of the furrowing reaction under widely varying temperature conditions. This work also indicated that the furrowing strength may be determined by the gelational state of the cortical cytoplasm in the region of the furrow. Consequently, it seemed important to ascertain how the furrowing strength of the eggs would be affected by the drug-induced changes in the plasmagel layer.

Action of mersalyl acid on furrowing (Arbacia). In these experiments the eggs were immersed in the mersalyl acid (2×10^{-3} M) sea water medium, equilibrated to the experimental temperature, starting at a time that was calculated to be 30 minutes prior to the appearance of the first furrows. The expected furrowing time, however, was not always predicted with complete accuracy, so that the actual time of immersion, prior to the pressure test, varied between 29 and 34 minutes. Shortly after the immersion was started the eggs were placed in the microscope-pressure chamber (which was maintained at the proper temperature) and kept under continuous observation as the time for cleavage approached. Then, at the time when 50% of the eggs showed at least incipient furrows, the pressure was built-up and maintained for 20 minutes. Finally, before releasing the pressure, a count of some 200–300 eggs was made to determine what percentage of the furrows had been aborted by the treatment.

Since incipient furrows are blocked by slightly lower pressure than are deeper ones (see Marsland, '50), the effectiveness of pressure in blocking the cleavage depends to some extent upon the timing of the pressure treatment. Consequently, when pressure is applied at the 50% furrowing time some of the eggs are less susceptible to blockage than are others. Accordingly it was necessary to select a definite arbitrary criterion. In these experiments a blockage of 70–

80% of the 200–300 eggs counted was adopted as the standard and not less than three experiments were performed for each of the experimental points recorded. Moreover, for each drug-test, usually two controls (one before and one after the drug-test) were run, using eggs derived from the same female.

The results of these Arbacia-mersalyl acid experiments are shown graphically in figure 7. Here it may be noted that the drug-treated eggs, judged by the pressure level required to block the furrows, display a distinctly lower furrowing strength than the untreated controls. In fact, the pressure differential at each of the two temperatures tested proved to be the same (approximately 1000 lbs./in.²) which is a further indication that the solational effects of pressure upon the normal and drug-weakened plasmagel systems are parallel (see fig. 5). Unfortunately the tests could be performed at only two temperatures, 20° and 25°C., since at 15° and 30°C., even under atmospheric pressure conditions only a

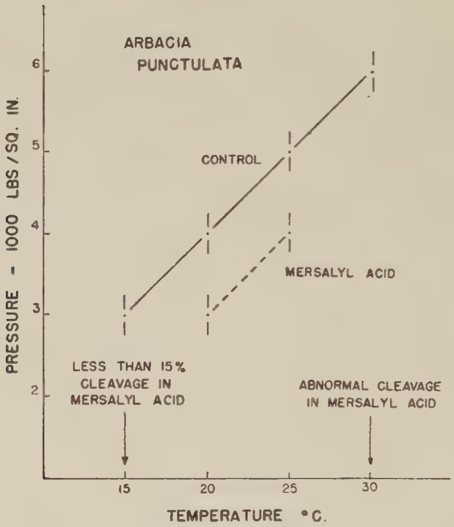

Fig. 7 The effect of 0.002 M. salyrgan (mersalyl acid) on the furrowing strength of the Arbacia egg. The minimum pressure adequate to prevent cleavage is plotted as a function of temperature.

small percentage of the drug-treated eggs came through first cleavage. Also, it should be recorded, early and late in the breeding season a few batches of eggs were found in which the furrowing strength was distinctly lower than that shown in the graph. Even in these batches, however, there was a distinct differential in the furrowing strength of the drug-treated and control samples.

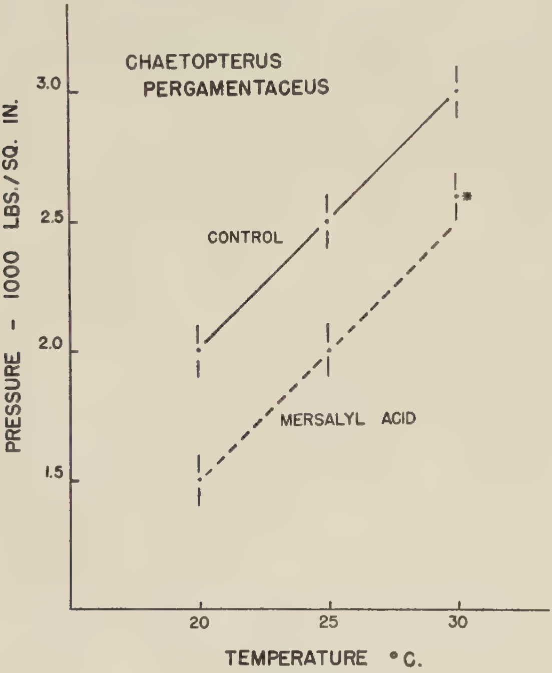

Fig. 8 Effect of salyrgan (0.002 M.) on the furrowing strength of the Chaetopterus egg. The minimum pressure required to block furrowing is plotted as a function of temperature. At the starred point (30°C.) the concentration was reduced to 0.001 M. since no cleavage occurred, even at atmospheric pressure, when the higher concentration was employed.

Action of mersalyl acid on furrowing (Chaetopterus). The Chaetopterus experiments followed the Arbacia pattern except that: (1) the immersion time (which did not prove to be very critical) was reduced to 20 minutes and (2) the pressure was applied at the moment when the polar lobe was plainly discernible in 50% of the eggs.

As may be seen in figure 8, the furrowing strength in Chaetopterus is distinctly lower than in Arbacia in that the furrow-inhibiting pressure at each different temperature is only half as great. However, the mersalyl acid effect for Chaetopterus is very definite, since blocking the cleavage of the drug-weakened eggs requires approximately 500 lbs. less pressure at each of the different temperatures studied.

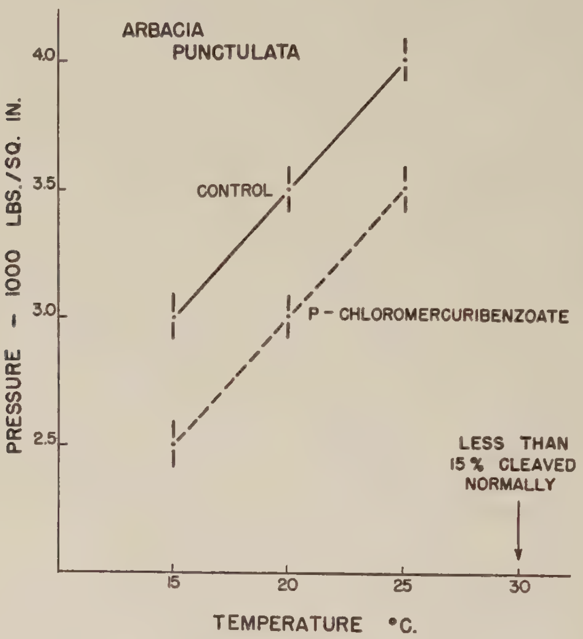

Fig. 9 Effect of 5×10^{-5} M. p-chloromercuribenzoate on the furrowing strength of Arbacia eggs. The minimum pressure required to block cleavage is plotted as a function of temperature for both the control and drug-treated eggs.

Action of p-chloromercuribenzoate on furrowing (Arbacia). These experiments resembled the other Arbacia experiments, except that: (1) Ca^{++}-Mg^{++}-free sea water served as the medium for both the control and the drug-treated eggs, and (2) the concentration of p-chloromercuribenzoate was 5×10^{-5} M.

Again the immersion period prior to the pressure treatment was standardized at 30 minutes and the pressure was built-up at the moment when furrows appeared in 50% of the eggs.

Figure 9 indicates that p-chloromercuribenzoate, compared to mersalyl acid, exerts a similar action. At each of the temperatures tested, the strength of the furrowing reaction was distinctly weaker in the drug-treated eggs. But in addition it seems apparent that the furrowing of eggs in Ca^{++}-Mg^{++}-free medium is less forceful than it is in normal sea water and both of these results appear to be related to the previously measured effects of the medium and the drug upon the gel structure of the egg cortex.

DISCUSSION

Significance of the sol-gel fluctuations in the cortical cytoplasm

A truly comprehensive interpretation cannot be given at present in regard to the functional significance of the complex series of very rapid and fairly drastic changes which occur in the gel structure of the egg cortex while the gamete nuclei are coming together and fusing and while the zygote is preparing for and consumating the first cleavage divisions. The studies of Heilbrunn ('20), Fry ('36), and E. B. Harvey ('41, '56), provide some very helpful data as to the time schedule of the events of syngamy and of the first cleavage mitosis in Arbacia. However, there are still some gaps and uncertainties in the schedule and as yet it is only possible to point out some very tentative correlations between the cortical gel changes and the physiological events.

The first gelational peak. This first build-up of the gel structure of the cortical cytoplasm (fig. 1) appears to be related to the development of the sperm aster and the approach of the gamete nuclei. As the gel strength rises in the cortex, there is also a marked increase in the viscosity of the deeper cytoplasm, as shown by the work of Heilbrunn ('20 and '28), and Fry and Parks ('34). However, the viscosity rise in the deeper cytoplasm appears to lag slightly behind the gelation of the cortex. Fry and Parks ('34) found that the viscosity rise in Arbacia eggs at 20°C. requires 15 minutes to reach a maximum, as compared to the 13 minute maximum of cortical gelation found in these experiments. However, the time from insemination to 50% first cleavage (at 20°C. ± 0.5) as reported by Fry and Parks ('34) for Arbacia eggs (late in breeding season) was 66 minutes. This represents a delay of some 10% compared to the usual value of 58–60 minutes found by other investigators (Loeb, '13; E. N. Harvey, '32; Hoadley and Brill, '37; and the present workers). Thus when the Fry timing is adjusted to the schedule used in the present experiments (by multiplying each time figure by a factor of 0.9) the gel strength rise of the cortex and the viscosity increase of the deeper cytoplasm are almost synchronous. Subsequently, however, the two curves separate radically, since the internal viscosity remains high for a longer period and falls off rather gradually (Heilbrunn, '20, '28; and Fry and Parks, '34).

The precise time at which contact between the gamete nuclei is achieved at 20°C. has not been determined unequivocally. The best evidence indicates, however, that this important event occurs between 12th–14th minute following insemination. E. B. Harvey ('41) working with Arbacia at 23°C. reported that the monaster reached a maximum development and that syngamy occurred at the 10th minute. This is equivalent to the 12th minute at 20°C., since Fry ('36) has shown that the timing of the stages of syngamy and mitosis between 15° and 25°C. is proportional to the total cleavage time. Also, the timing of Fry and Parks ('34), adjusted to the normal 59 minute cleavage cycle, indicates that the nuclei make contact 13–14 minutes after insemination. It seems clear, therefore, that temporally, at least, the first post-insemination fortification of the cortical gel layer and the first rise in the "cytoplasmic viscosity" are both closely related to the development of the monaster and to the coming together of the male and female pronuclei.

It is conventional to assume that the sperm aster is in some way responsible for bringing the gamete nuclei together and on this basis it is tempting to postulate that the gelation of the egg cortex is likewise involved. Pease ('41) has shown that the amphiaster and spindle represents gel structures which are susceptible to dissolution under hydrostatic compression. Thus it seems possible that the surface changes initiated when the sperm contacts the egg give rise to a rapid gelation of the cortical cytoplasm and that this gelational tendency quickly spreads to the deeper cytoplasm, where it leads to the development of the sperm aster. Perhaps, indeed, the cortical gel provides anchorage and orientation for the developing aster. However, this and further speculation cannot be fruitful until more is known about the complex series of events and processes that follow so quickly upon one another after the sperm makes contact with the egg.

The second post-insemination gelational peak. Following the time of contact between the pronuclei the cortical gel strength drops quickly, reaching a moderately low minimum 5 minutes later, at the 18th minute after insemination (see fig. 1). Then a rapid gelation sets in. This reaches a second short-lived peak at the 23rd minute, after attaining a level approximately equal to the first peak.

These rapid shifts in the sol-gel equilibrium of the cortical cytoplasm are difficult to interpret in terms of any known cytological changes in the egg. During the period in question, apparently, the gamete nuclei consumate their fusion and the monaster maintains itself with only a slight degree of fading (see Fry, '36; and Fry and Parks, '34). Also during this period, it seems likely, the centrosomes start moving apart toward their final position on opposite sides of the zygote nucleus. However, according to the observations of E. B. Harvey ('41, '56) the streak stage (which she interprets as representing the full separation of the centrosomes) does not reach a maximum until the 25th minute, even in eggs developing at 23°C. At 20°C. the calculated time at which the centrosomes achieve full separation would be the 30th minute. In other words, the second maximum gelation of the egg cortex seems to anticipate the full separation of the centrosomes by approximately 7 minutes.

One may speculate that the monaster becomes involved in the separation of the centrosomes subsequent to syngamy and that the restoration of a strongly gelled cortex is necessary for anchorage and orientation. This view fits in with the observation that the monaster of Arbacia during this period becomes elongate and develops two vague focal centers, corresponding to the separating centrosomes (see Fry and Parks, '34). However, with this hypothesis it is difficult to understand why the higher gelational state of the cortex does not persist until the centrosomes have been completely separated. Therefore it seems better to await further information before attempting a more definite interpretation of these results.

The final rise of the first division cycle. The extensive experiments of Marsland and co-workers have shown that the very strong gelation of the cortical cytoplasm plays a dominant role in determining the strength of the furrowing reaction at telophase and this aspect of the problem will be discussed later, in relation to the action of the sulfhydryl drugs. Here, however, it should be pointed out that the gelational state of the cortex reaches a very high level of structural strength somewhat before the onset of the prophase of the first cleavage mitosis. According to the adjusted figures of Fry and Parks ('34) and of Harvey ('41) the first really definite cytological evidence of the presence of the amphiaster of the first division does not appear (at 20°C.) until 40–42 minutes after insemination, whereas the mitotic cortical gelation reaction reaches its high plateau some 6–7 minutes earlier, at 33–34 minutes.

Viewing the sol-gel changes of the first division cycle as a whole, it seems evident that each of the cytological events of this period is anticipated by a definite build-up of the gel structure of the egg cortex. To be sure the degree of anticipation with reference to the development of the monaster and the achievement of syngamy is very slight compared to that which is related to the separation of the centrosomes and the appearance of the amphiaster, but nevertheless it appears to be definite. Moreover, the development of a strongly gelled structure, which persists in the cortex of the fertilized egg all through the cleavage period, indicates that this part of the cytoplasm may have a role to play during all of the mitotic phases.

Gelational changes of the second division cycle. Precise data as to the timing (at 20°C.) of the cytological events of the second cleavage division of the Arbacia egg are not available. Nevertheless, it seems probable that the first post-furrowing build-up of the cortical gel structure (fig. 1) is related to the movement of the centrosomes to opposite sides of the re-constituted nuclei of the blastomeres; and that the second gelational rise is related to the onset of the second cleavage. At present, however, the degree, if any, to which these cortical changes may anticipate the internal cytological events remains uncertain.

Comparison with earlier results. The sol-gel changes shown in figure 1 differ from those reported by Brown ('34), mainly in regard to the occurrence of a second gelational peak in the early part of the first division cycle. The detection of such a rapid fluctuation in the gelational state, apparently, requires that measurements be made at very short (1 minute) intervals, under meticulously controlled temperature conditions. Brown's excellent pioneer experiments, on the other hand, were carried on at room temperature and the intervals between measurements were not recorded. Moreover, the low centrifugal force (7200 × G) employed by Brown was not adequate to give a sharp and definite end-point, except in areas where the gel strength was low. Consequently, it may be said that the present experiments represent a refinement and extension of the earlier work and that essentially the two sets of data are in good agreement.

Energetics of cortical gelation in relation to cytokinesis

General viewpoint. The extensive evidence now available indicates that the energy expended by the egg cell, when it undergoes furrowing and divides itself into blastomeres, is derived from an inherent contractility of the cortical plasmagel layer (see Marsland and Landau, '54). At telophase, apparently, this contractility becomes localized in the region of the developing furrow so that cleavage results from a constriction of the cell in this region. Thus metabolic energy, which the cell diverts into the endothermic process of gelation, finally appears as useful mechanical energy when the cell system contracts and performs the work of cleavage.

Action of sulfhydryl drugs. The study of sulfhydryl-blocking agents, such as salyrgan and p-chloromercuribenzoate, in relation to plasmagel structure and activity, stems from the hypothesis that the cortical gelation reaction may receive energy from the adenosine triphosphate system of the cell. These particular drugs have a very marked inhibiting effect upon various ATP-ase systems, as has been shown for salyrgan by the extensive work of H. H. Weber and coworkers (see Weber, '55a, b; Weber and Portzehl, '52 and '54; Hoffmann-Berling, '53 and '54a, b, c; and Kuschinsky and Turba, '50a, b; and '51), and for p-chloromercuribenzoate by the thorough studies of Barron and associates (see Singer and Barron, '44). However, it also seems evident that the sulfhydryl drugs have a very definite though perhaps a less specific effect upon a wide variety of other metabolic enzymes (Barron and Singer, '45 and Barron, '51).

The importance of ATP as an energy source in muscle tissues (see Weber, '55a, b) naturally suggested that this metabolite might contribute energy to the sol-gel cycle in cells generally. And now, indeed, considerable evidence in this direction has begun to come from a number of sources. Runnstrom and Kriszat ('50) and Kriszat and Runnstrom ('51) have shown that egg cells immersed in ATP solutions become more resistant to hypotonic cytolysis, which seems to be related to a gelling effect upon the cytoplasm. Kriszat ('49 and '50) found that ATP treatment has a very marked effect upon the form and movement of *Amoeba proteus.* Loewy ('52) has extracted an actomyosin type of protein from the amoeboid slime mold, *Physarum polycephalum,* and has shown that this preparation undergoes large changes in its gelational state in the presence of ATP and related compounds. Hoffmann-Berling and Weber ('53) and Hoffmann-Berling ('54a) have shown that glycerol-extracted fibroblasts display a quick and forceful contraction of their elongate pseudopodia when treated with ATP solutions and that this remarkable contraction can be stopped quickly and reversibly by the addition of salyrgan to the medium. Hoffmann-Berling ('54c) also showed that fibroblasts, killed by glycerol-water extraction just at the beginning of telophase, when shallow cleavage furrows had just appeared, display a remarkable deepening of the furrows, virtually to the point of complete cleavage, when appropriate concentrations of ATP are added to the immersion medium. And finally, Landau, Marsland and Zimmerman ('55) have reported that the eggs of both Arbacia and Chaetopterus show a very distinct increase in the strength of the furrowing reaction as a result of adding ATP (0.0005 M) to the sea water; and that this improved furrowing performance seems to be related to a demonstrable strengthening of the cortical plasmagel layer by the ATP treatment (cf. Wicklund, '54).

The results of the present experiments give further support to the hypothesis that the ATP system contributes energy to the cortical gelation reaction and that this energy later becomes available for mechanical work during the furrowing process. In the case of salyrgan, the drug definitely delays and reduces the building up of the cortical gel structure which is associated with the first mitosis (fig. 4). At the same time, as may be seen in figure 5, the drug-weakened plasmagel of the egg displays the same susceptibility to pressure solation as does the normal system. Consequently it seems most significant that the furrowing strength of the drug-treated eggs proves to be correspondingly lower. This is evident in figures 7 and 8, which show that the level of pressure required to stop furrowing is reduced in salyrgan-treated eggs by about 1000 lbs./in.^{2} at each temperature for Arbacia, and about 500 lbs./in.^{2} for Chaetopterus.

In the case of the p-chloromercuribenzoate experiments, the effects upon the gel system and on the furrowing performance appear to be quite similar. As may be seen in figure 6, the plasmagel system of the fertilized Arbacia egg becomes weaker as a result of the treatment with p-chloromercuribenzoate, and at the same time the weakened system retains its susceptibility to further solation when high pressures are applied. Moreover, these effects upon the gel system display a close relationship to the observed action of this drug upon the furrowing strength, which is shown in figure 9. Here it may be seen that the level of pressure required to abort furrows, at each of the different temperatures measured, proves to be some 500 lbs./in.^{2} lower, as compared to control eggs.

Perhaps the work of Wilson ('51), which also dealt with the Chaetopterus egg, should be considered here. Wilson found that the gelational strength of the cortical cytoplasm in the Chaetopterus egg tends to fall markedly starting about 15 minutes prior to furrowing and to remain low during all of the first cleavage mitosis. However, the criterion used by Wilson in determining cortical gel strength was the occurrence of a break in the continuous line up of cortically embedded granules. This scarcely seems adequate as an index of the state of the cortex as a whole. In fact the development of a definitive furrow seems to depend upon a preliminary weakening of the gel structure at the prospective poles as has been emphasized by Marsland and Landau ('54). Moreover, such a localized weakening which is essential to the establishment of a structural differential in the plasmagel system, might be expected to occur quite some time before furrowing in Chaetopterus, since this egg always produces a *polar lobe* before the true cleavage furrow appears (cf. Heilbrunn and Wilson, '48 and '55).

Mode of action of the sulfhydryl drugs. At present, it is not possible to conclude that the action of the sulfhydryl reagents is solely upon the enzymes of the ATP system in the egg. In fact there seem to be a number of important metabolic enzymes in which catalytic activity depends, at least partly, upon the pattern of –SH radicals in the protein moiety of their structure (see Singer and Barron, '45; Barron and Singer, '45; Barron, '51; and Putnam, '53a, b). Nevertheless, in view of the following considerations, it seems probable that the drugs used in these experiments may act primarily upon the ATP system.

Singer and Barron ('44) have shown that the ATP-ase activity of myosin is abolished by p-chloromercuribenzoate and restored by the addition of glutathione. Also Barron ('53) has reported that ATP-ase is particularly susceptible to sulfhydryl blocking agents. It is one of a small group of enzymes in which the loss of activity is proportional to the concentration of the sulfhydryl reagent, which indicates that a full integrity of the –SH groups is necessary for catalytic activity.

Perhaps the best indication that the ATP system participates directly in contractile processes even in relatively undifferentiated cells is provided by the cell model experiments of Weber and Portzehl ('54), Portzehl ('52 and '54), and Hoffmann-Berling ('53 and '54a, b, c). In glycerol-water extracted cells, used by these investigators, virtually all the crystalloids, globular proteins, ordinary enzymes, etc. are removed and the cell membranes are destroyed. Practically all that remains is a skeleton of fibrous protein. This protein residue behaves essentially like the thread models which these workers have prepared from actomyosin, and like their glycerol-extracted muscle-fiber models, upon the addition of appropriate concentrations of ATP, the cell framework contracts forcibly, but the contraction stops abruptly when salyrgan is added to the system. Moreover, this inhibiting action of salyrgan is counteracted by cysteine, which indicates that –SH groups play an important role in determining the activity of the system. Undoubtedly the action of sulfhydryl drugs in the cell models is a direct one upon the ATP system. Accordingly, there is a valid basis for assuming, as a working hypothesis, that the action of these drugs in weakening the cortical gel structure and reducing the furrowing strength of the living eggs is accomplished on a similar basis.

There remain, however, some puzzling questions in regard to precise action of the drugs on plasmagel structure and activity. In muscle, of course, the myosin component serves a dual role. Not only does it represent an essential part of the contractile system, but also it is responsible for the ATP-ase activity of muscle, — as was first revealed by Engelhardt and Ljubimowa ('39). It seems possible, therefore, that the fibrous protein or proteins which participate in the formation of the contractile gel structures of other cells may likewise serve in such a dual capacity. In fact this viewpoint seems almost inescapable in light of the cell model experiments of the Weber group, since little if anything alse remains in these "cells" following the drastic glycerol-extraction process.

On the basis of the foregoing viewpoint, it becomes difficult to separate the effects of the –SH reagents upon inter- and intra-molecular linkages — which determine gel structure and contractility — and the effects of these agents upon ATP-ase activity. The work of Bailey and Perry ('47) indicated that the combining of actin with myosin takes place through the –SH groups of the myosin and at the same time Singer and Barron ('44) show that the ATP-ase activity of myosin is abolished by p-chloromercuribenzoate and restored by the addition of glutathione. Thus it would appear that the –SH drugs may interfere with the contractile process not only by deranging the structure of the enzyme which provides energy for the reaction, but also by directly blocking the formation of intermolecular linkages necessary to the structure of the contractile elements. In other words, with reference to the present experiments, we may postulate that the sulfhydryl drugs (salyrgan and p-chloromercuribenzoate) achieve a reduction in the furrowing strength of the dividing eggs in at least one, but more probably two ways: — (1) by interfering with the ATP-ase activity of the cell, — and (2) by weakening the gel structure of the cortical cytoplasm more directly through the disruption of intermolecular linkages between the component protein units of the system.

The situation seems further complicated by the fact that ATP itself may have a double role to play, as is indicated by the work of Weber and his group. According to Weber and Portzehl ('52 and '54), and Weber ('55a, b), ATP serves as a contracting agent in muscle fiber models only so long as the hydrolytic splitting of the high energy phosphate linkages is actively progressing. But ATP also seems to serve as a "plasticizing" (or relaxing) agent when the hydrolysis is in abeyance. Moreover, these ideas are being extended by Hoffmann-Berling ('53 and '54a, b, c) to pseudopodial contraction, to the furrowing process and even to the anaphase contraction of spindle fibers, in tissue-cultured fibroblasts and other cells.

Significance of the premature furrowing phenomenon

At present no satisfactory interpretation of this strange reaction can be offered. It seems to indicate the existence in the egg of some sort of factor, substance or structure, which is susceptible to displacement under the rather drastic centrifugal conditions used, and which is capable of initiating a local contraction in the cortical plasmagel layer. It is true, to be sure, that the nucleus and affiliated parts of the mitotic apparatus are displaced, in the Arbacia egg, into the light half of the cell where the premature abortive furrows always appear; but it is likewise true that such furrows only appear under conditions which also produce a very marked dislocation of the materials of the strongly gelled cytoplasm of the egg cortex. Moreover, according to the normal schedule of development, premature furrowing occurs most frequently some

3–4 minutes before the zygote nucleus begins to show signs of prophase activity.

SUMMARY

Pressure-centrifuge measurements of the structural state of the gelated cortical cytoplasm (plasmagel layer) of the Arbacia egg were made at one minute intervals from insemination to second cleavage. At the temperature employed (20 ± 0.3°C.), the first division (50% furrows) occurred at 59 ± 2 minutes, and the second division came 33 ± 1 minutes later. Both the centrifugal force (41,000 × G.) and the pressure (8,000 lbs./in.^{2}) were quite high. Consequently a standard displacement of the red chromatophores in the cytoplasmic cortex was achieved within relatively short (50–200 seconds) periods of centrifugation.

The data show that fertilization initiates a complex series of gelational changes in the cortical cytoplasm of the egg. In the first division cycle, there is a rapid, initial strengthening of the gel structure which reaches a high but short-lived peak in 13 minutes. After this, there are two other peaks at 23 and 33 minutes, respectively. The intermediate peak resembles the initial one as to magnitude and very short endurance, but the final rise is larger and it persists until the first furrows have been completed. In the second division cycle, only two peaks occur. The first, which occurs at the 69th minute, is short-lived, but the second, which starts at the 85th minute, persists until the furrows have completed the second division.

Two sulfhydryl-blocking agents, namely salyrgan (mersalyl acid) and para-chloromercuribenzoate, were tested as to their effects upon the plasmagel structure and furrowing capacity of the eggs of *Arbacia punctulata* and *Chaetopterus pergamentaceus.*

In the case of salyrgan ($2\text{–}4 \times 10^{-3}$ M), it was found that the drug definitely delays and reduces the gelational increases of the first division cycle of the Arbacia egg. This effect is especially clear with reference to the final peak of gelation, which is concurrent with cleavage. Moreover, the furrowing

strength of both kinds of eggs is plainly reduced by the salyrgan treatment, since the pressure required to block furrowing is reduced — by 500 lbs./in.^{2} at each temperature (20°, 25° and 30°C.) for Chaetopterus — and by 1000 lbs./in.^{2} at 20° and 25°C., for Arbacia.

Similarly for para-chloromercuribenzoate (5×10^{-5} M), the drug was found to have a weakening effect upon the gel structure of the cortical cytoplasm of the Arbacia egg, measured just prior to the onset of cleavage. Also the drug produced a distinct weakening of the furrowing strength, since the pressure required to block cleavage was reduced by 500 lbs./in.^{2} at each of the temperatures (15°, 20° and 25°C.) tested.

The significance of the gelational changes in the structure of the cortical cytoplasm is discussed in relation to the approach of the pronuclei, syngamy and the cleavage mitosis.

A general interpretation of the data is attempted in terms of the plasmagel contraction hypothesis of cytokinesis. The results indicate that metabolic energy from the ATP system of the egg initially goes into the formation of the cortical gel structure, and then later appears as mechanical energy, when the gel contracts during the furrowing process.

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OBSERVATIONS ON THE AFFECT OF METALLIC MERCURY UPON SOME MICROORGANISMS[1]

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FOUR FIGURES

Although it has long been known that metallic mercury can exert a toxic influence on living organisms (Pennell and Weatherley, 54; Stock, '42), many workers have made metabolic studies using a mercury electrode (Blinks and Skow, '38; Dutton and Manning, '41; Moore and Duggar, '49; Petering and Daniels, '38; Winzler, '41) for measuring oxygen tension, in which metallic mercury comes into more or less intimate contact with the organism. These workers had recognized the danger of mercury poisoning and had taken precautions to ensure that their results were not being affected by the presence of mercury. Most organisms studied, including yeast (Petering and Daniels, '38; Winzler, '41), *Chlorella* (Dutton and Manning, '41; Moore and Duggar, '49; Petering and Daniels, '38), *Ulva* (Blinks and Skow, '38), and the higher plants *Potamogeton* and *Ricinus* (Blinks and Skow, '38), were sufficiently resistant to show no noticeable affect of mercury under the conditions used. However, in at least one case, that of the diatom, *Nitzschia closterium* (Dutton and Manning, '41) there was a pronounced toxic influence of mercury.

Because mercury electrodes have been used so successfully to study the oxygen exchange of a considerable variety of organisms, there is, perhaps, not sufficient appreciation of possible deleterious affects of mercury upon metabolism. Pennell

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and Weatherley ('54) have recently shown that merely exposing leaf discs of deadly nightshade or lettuce to mercury vapor will greatly inhibit the active uptake of sucrose by these tissues. In this paper the affect of metallic mercury upon several phases of the metabolism of an extremely sensitive organism, the green alga, *Scenedesmus obliquus,* Strain D_3, is described. Its affects are similar to those of an "uncoupling agent" like dinitrophenol (Simon, '53). The rate of respiration increases to as much as 300% of the control value, while the photosynthetic rate declines; in addition, the coupling between the oxyhydrogen reaction (Gaffron, '42) and reductive fixation of carbon dioxide is apparently broken. Observations on the mechanism of action of the mercury indicate that an oxidized form of the mercury, probably the mercuric state, causes the observed affects on metabolism, and that the oxidation occurs within the cell. Affects of mercury on several other organisms are also described.

Affect on respiration and photosynthesis of Scenedesmus D_3

The algae used in the studies described in this paper were grown bacteria-free at 28°C. in 500 ml of nutrient solution in broad bottomed culture vessels which were in continuous gentle oscillation on a shaker and with 4% carbon dioxide in air bubbling through the liquid. Fluorescent lamps provided an illumination of about 850 foot candles at the culture vessel. The medium contained macroelements in the proportions used by Allen ('55), and to each liter was added 1 ml of the iron solution that he described. The minor elements added were tungsten, molybdenum, boron, bromine, iodine, manganese, zinc, cadmium, cobalt, copper, nickel, chromium, vanadium, and aluminum.

The mercury had been redistilled under vacuum and was then further put through several purification procedures: (1) bubbled violently with air, (2) passed through aqueous 10% potassium hydroxide as fine droplets, (3) passed through dilute nitric acid as fine droplets, (4) washed with distilled water.

The original observation that led to a study of the affect of mercury was the following: The endogenous rate of oxygen uptake by *Scenedesmus obliquus,* strain D_3, when measured with a dropping mercury electrode (Petering and Daniels, '38), was more than double the rate of oxygen uptake measured by Warburg manometry. In pursuit of the cause of this anomaly, some metallic mercury was added to the cell suspension in the Warburg vessel, whereupon, as soon as readings could be started, a marked stimulation of the respiratory rate over that of the control was observed. If the mercury was left in the suspension, the stimulation gradually changed over to an inhibition which might become practically complete. Larger amounts of mercury in the suspension cause a faster development of the stimulation and subsequent inhibition. Also the thinner the cell suspension, the faster is the development of the mercury effects. With sufficiently thin suspensions and enough mercury present, there may be almost complete inhibition of oxygen uptake as soon as manometric readings are started. In 1941, Dutton and Manning had also reported that, with the diatom *Nitzschia closterium,* thinner cell suspensions favor the appearance of toxic affects of metallic mercury.

Scenedesmus D_3 is so sensitive that, even if 1.5 gm of mercury are put in the side arm of a Warburg vessel, with no contact between the cell suspension and the mercury, marked effects on the metabolism appear within less than an hour due to diffusion of the vapor through the gas phase. Figure 1 shows the affect of mercury in the side arm on the endogenous oxygen consumption and photosynthesis of *Scenedesmus* D_3 suspended in Warburg no. 9 carbon dioxide buffer (Warburg, '19) containing .01 M KCl.[2] Similar effects were observed in Warburg no. 9 buffer lacking KCl, in Warburg no. 1 buffer containing .01 M KCl, in .05 M phosphate buffer of pH 6.5

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[2] KCl was included because it was present in the suspensions studied in the dropping mercury electrode and also because, as is discussed later, chloride ion has important effects upon the equilibria of mercury ions.

with or without .01 M KCl, in .05 M acid phosphate with .01 M KCl, and in culture medium. The characteristic acceleration and subsequent inhibition of respiration occurs with 10% potassium hydroxide in the center well or with 4% CO_2 present in the gas phase.

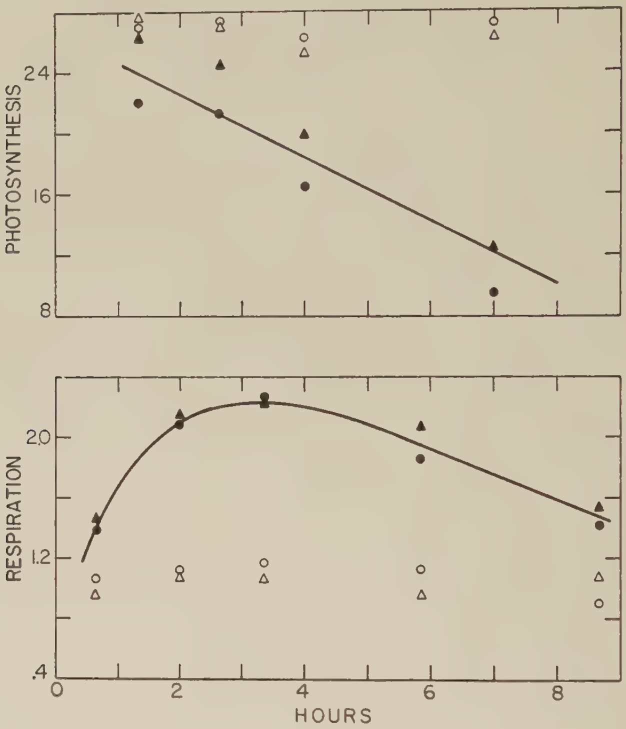

Fig. 1 Affect of mercury vapor on respiration and photosynthesis of *Scenedesmus* D_3. Ten and eight-tenths microliters of algae suspended in 1 ml of Warburg no. 9 carbon dioxide buffer (Warburg, '19) (Na:K::65:35 and containing .01 M KCl) in main compartment of Warburg vessel. The abscissa is time in hours and the ordinate is oxygen uptake rate (respiration) or oxygen production rate in the light (photosynthesis) in cell volumes per hour. Periods of photosynthesis were alternated with periods of respiration. The filled circles are for metabolic rates with 1.5 gm of mercury in the side arm; the filled triangles are for rates with an amount of red mercuric oxide in the side arm of about the same volume as 1.5 gm of mercury; the open triangles are for rates with a similar volume of mercuric chloride powder in the side arm and the open circles are for the control with no mercury compound in the vessel. T = 28.3°C.

Although the presence in the Warburg vessel side arm of any one of the solid mercury compounds: mercuric chloride, mercuric nitrate, mercuric sulphate, or mercurous chloride, had no effect on metabolism, red mercuric oxide had an effect almost identical to metallic mercury (see fig. 1). This is explicable in the light of the observation by Stock ('34) that the vapor pressure of mercury over red mercuric oxide can be enormously greater than one would predict from thermodynamic data and not far different from the vapor pressure of mercury over metallic mercury. (From thermodynamic data [Latimer, '38] one calculates a partial pressure of mercury over red mercuric oxide in air of the order of 10^{-13} mm of mercury, while the vapor pressure of mercury over metallic mercury is of the order of 10^{-3} mm ofmercury.)

The degree of stimulation of the respiration of *Scenedesmus* by mercury is quite similar to the degrees of stimulation of respiration described for dinitrophenol and related compounds acting on other organisms (Simon, '53). In favorable cases the mercury stimulated respiration approaches 300% of the control rate, but in other cases the stimulaton may be practically undetectable. Also, as in the case of the dinitrophenol group of compounds, there appears to be an inverse correlation between the rate of the control respiration and the per cent of stimulation (Simon, '53). Conditions conducive to a high rate of control respiration resulted in lower percentages of stimulation and *vice versa.* For instance, cells grown under an iron deficiency had a high rate of control respiration and an almost unnoticeable stimulation, while cells grown to a low cell density under relatively low light intensity had a low rate of control respiration and a high percentage stimulation.

Affect on the oxyhydrogen reaction of Scenedesmus D_3

Scenedesmus D_3, after anaerobic adaptation to hydrogen, is able in the dark, to perform the oxyhydrogen reaction (reaction A), coupled with the fixation and reduction of carbon dioxide (reaction B), in which the ratio of carbon dioxide consumed to oxygen consumed is normally 1/2 (Gaffron, '42; Horwitz, '57):

(A) $O_2 + 2H_2 \rightarrow 2H_2O$

(B) $1/2\ CO_2 + H_2 \rightarrow 1/2\ (CH_2O) + 1/2\ H_2O$

(C) $O_2 + 1/2\ CO_2 + 3\ H_2 \rightarrow 1/2\ (CH_2O) + 2\text{-}1/2\ H_2O$

Depending on the way in which it is applied, metallic mercury may completely stop any manifestation of the oxyhydrogen reaction or, it may merely stop the reaction to an extent which corresponds to the elimination of the coupled carbon dioxide fixation part (reaction B). Either 1.5 gm or .1 gm of metallic mercury in the cell suspension in a Warburg vessel (containing 1.5–1.9 µl of *Scenedesmus* D_3 in 1 ml total volume of liquid; the suspending medium is .05 M phosphate buffer of pH 6.5 with .01 M KCl) during the adaptation period of 9–13 hours at 28.3°C. practically completely eliminates any trace of the oxyhydrogen reaction. However, if under the same conditions, the mercury is either kept in the side arm, out of direct contact with the liquid phase, or else is tipped into the liquid phase only immediately before the initiation of the reaction, then it appears that only reaction B is eliminated.

The oxyhydrogen reactions were run in Warburg respirometers under conditions in which oxygen tension was not limiting (Horwitz, '57), and they were usually run until all the oxygen present was consumed and the reaction ceased. If the gas quotients given by reactions A, B, and C are assumed, therefore, to hold throughout the course of the reaction, one can calculate, either from the initial rate of pressure change and the rate of the control reaction, or from the total pressure change at the completion of the reaction and the known amount of oxygen present intially, the molar ratio (r) of carbon dioxide consumed to oxygen consumed. If the calculation is made from the initial rate of pressure change, then, in order to calculate the carbon dioxide: oxygen ratio (r) in a disturbed or uncoupled reaction, it is necessary to assume that, in the undisturbed control reaction, $r = 1/2$. In any case, r is calculated assuming that reaction (A) proceeds undisturbed. These assumptions seem to be justified on the basis of prior studies of the oxyhydrogen reaction (Gaffron, '42; Horwitz, '57), and also because they result in consistent answers.

In order to calculate r, the carbon dioxide: oxygen ratio, from either an initial rate measurement or from the total pressure change of a completed reaction, one can use the same formula, since it was assumed that the gas quotients given by reactions (A), (B), and (C) hold throughout the course of the gas uptake. The formula is:

$$r = -\frac{P/y - 3/k_{O_2}}{1/k_{CO_2} + 2/k_{O_2}}. \tag{1}$$

In calculating r from the total pressure change, P = total gas uptake in millimeters of Brodie solution and y = total amount of oxygen initially present in microliters; in calculating from initial rate, P = initial rate of pressure change in millimeters of Brodie solution per hour and y = rate of oxygen uptake in microliters per hour in the normal, control reaction. The symbols k_{O_2} and k_{CO_2} are respectively the ordinary Warburg vessel constants for oxygen and carbon dioxide. In table 1, there appear data calculated from equation (1).

These data indicate that a limited exposure of hydrogen adapted *Scenedesmus* D_3 to metallic mercury results in the uncoupling of carbon dioxide fixation and reduction (reaction B) from the oxyhydrogen reaction (reaction A). In this respect a limited exposure to mercury acts like dinitrophenol on the oxyhydrogen reaction (Horowitz, '57; McFadden and Atkinson, '55), and is also analogous to the uncoupling inhibition by dinitrophenol of a variety of energy requiring processes (Simon, '53).

Affect of mercury on some other organisms

The affect of mercury on the respiration of nine other organisms was also studied. Respiration was measured as the rate of oxygen consumption in a Warburg vessel with 10% KOH on filter paper in the center well. A control vessel, without organisms, containing 10% KOH on filter paper and mercury showed only negligible gas uptake. The extent of the

TABLE 1

The carbon dioxide: oxygen ratio calculated under various conditions

CONDITIONS	k_{O_2}	k_{CO_2}	INITIAL RATE: PRESSURE CHANGE	INITIAL RATE: O_2 UPTAKE	O_2 INITIALLY PRESENT	TOTAL GAS UPTAKE	r FROM INITIAL RATE	r FROM TOTAL PRESSURE CHANGE
Control	1.33	1.49	$15\,\frac{\text{mm Brodie}}{\text{hour}}$	$4.5\,\frac{\mu l}{\text{hour}}$	.16%	71 mm Brodie	+ .50 (assumed)	+ .52
1.5 grams Hg always in side arm	1.33	1.49	$11\,\frac{\text{mm Brodie}}{\text{hour}}$		.15%	50 mm Brodie	+ .09	+ .11
.1 gram Hg tipped into liquid at start of reaction	1.36	1.52	$9\,\frac{\text{mm Brodie}}{\text{hour}}$		.14%	43 mm Brodie	— .09	+ .03
.1 gram Hg tipped into liquid 16 minutes before start of reaction	1.33	1.49	$10\,\frac{\text{mm Brodie}}{\text{hour}}$		.17%	52 mm Brodie	— .02	.00
.1 gram Hg tipped into liquid 99 minutes before start of reaction	1.33	1.49	$10\,\frac{\text{mm Brodie}}{\text{hour}}$		.14%	47 mm Brodie	— .02	+ .10

investigation of these organisms was such that only a relatively crude qualitative comparison to *Scenedesmus* D_3 is possible. Seven of the organisms showed effects of mercury, but only one, *Bacillus cereus* var. *mycoides,* appears to approach *Scenedesmus* in sensitivity.

Three green algae, *Stichococcus bacillaris, Chlorella pyrenoidosa,* and *Ankistrodesmus Braunii,* were tested with mercury in the side arm. All showed stimulations of endogenous respiration, but the effects were smaller and slower in appearing than is usually the case with *Scenedesmus* D_3.

Two bacteria were tested. The endogenous respiration of *Escherichia coli,* strain B/R, was unaffected by mercury in the side arm, even after 12½ hours exposure. With mercury in the cell suspension though, the endogenous respiration of *Bacillus cereus* var. *mycoides* was soon completely inhibited; at no time did an accelerated respiration appear.

With mercury in the cell suspension, *Saccharomyces cerevisiae* developed a stimulated endogenous respiration that reached 250% of the control. Mercury also increased the respiration rate in the presence of glucose, and increased the total amount of absorbed oxygen that could be attributed to the oxidation of a small amount of glucose. In this case again mercury acted like the dinitrophenol group of compounds in that it apparently uncoupled glucose assimilation from an energy yielding process (Simon, '53).

During an exposure of leaf discs of two higher plants, *Nasturtium sp.* and *Phaseolus vulgaris,* to mercury in the side arm for 21 hours, marked inhibitions of the endogenous respiration occurred. At no time was a stimulation of the respiration observed.

Exposure of the fruit fly, *Drosophila repleta,* to mercury in a Warburg vessel for 21 hours caused no significant change in its rate of respiration

Mechanism of action

The influence of metallic mercury on metabolism has been attributed to the formation of an oxidized species (Stock, '34;

Stock, '42) in the presence of oxygen. That air promotes the development of toxic effects in *Scenedesmus* D_3 from metallic mercury was demonstrated in an experiment in which algae were exposed to mercury aerobically and anaerobically. Two milliliters of a .33% suspension of *Scenedesmus* D_3 in Warburg no. 9 carbon dioxide buffer containing .01 M KCl were put into the main compartments of each of two Warburg vessels. The vessels were of a type previously described (Gaffron, '42) with a side arm that could be sealed off from the main compartment. One and one-half grams of mercury were contained in each of the side arms, which were initially sealed off. After one vessel and its mercury containing side arm had been thoroughly flushed with prepurified nitrogen, both vessels were opened to their mercury containing side arms and shaken in the dark for 130 minutes. At the end of this time the rate of photosynthesis was measured in both vessels exposed to mercury and also in a control vessel containing no mercury. The results are shown in table 2.

TABLE 2

Affect of air on the development of mercury poisoning

T = 28.3°C.

CONDITIONS	RATE OF OXYGEN PRODUCTION
Atmosphere air; no mercury present	24.8 cell volumes/hour
Atmosphere N_2; mercury in side arm	26.7 cell volumes/hour
Atmosphere air; mercury in side arm	13.3 cell volumes/hour

The influence of air on the development of mercury poisoning might be explained by the oxidation of some of the liquid metallic mercury to HgO which then diffuses over to the cell suspension. This is unlikely, however, on the basis of the observation by Stock ('34) that the mercury present in the air over pure HgO is Hg and not HgO. More direct evidence of this is obtained from experiments, described below, with the chelating agent ethylenediamine tetraacetate, which indicate that the mercury oxidation which gives rise to a toxic agent occurs within the cell.

In support of the idea that an oxidized form of mercury, probably the mercuric form, is the agent responsible for the toxicity of metallic mercury, we observed that $HgCl_2$ at suitable concentrations can cause a large stimulation of endogenous respiration in both *Scenedesmus* D_3 and in *Chlorella pyrenoidosa*. This is like the effect of metallic mercury, and, like the stimulation due to metallic mercury, the degree of stimulation seems to be related inversely to the rate of the control respiration. Figure 2 shows data on the affect of $HgCl_2$ on respiration. Figure 3 shows data on the affect of p-chloromercuribenzoate on the endogenous respiration and photosynthesis of *Scenedesmus* D_3. A respiratory acceleration is also evident here, but the affects on metabolism take many hours to develop.

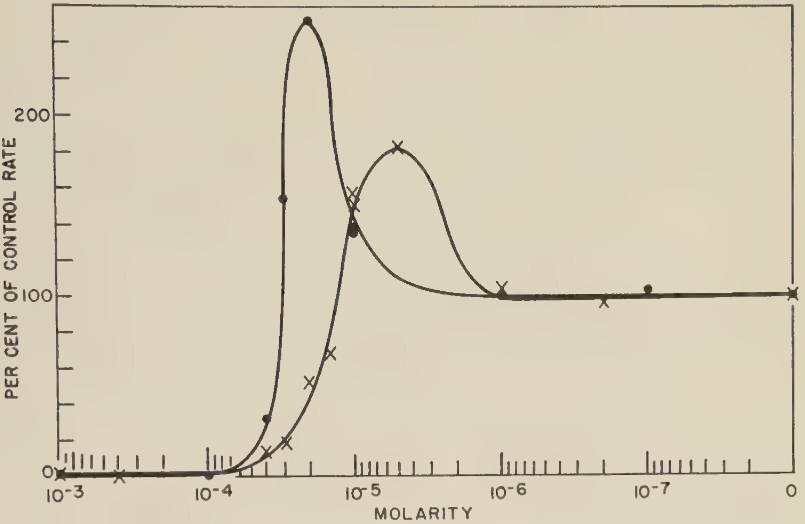

Fig. 2 Affect of mercuric chloride on respiration of *Scenedesmus* D_3 |X|, and *Chlorella pyrenoidosa* |●|. Suspending medium is .05 M phosphate of pH 6.5 with .01 M KCl. Abscissa: molarity of mercuric chloride on a logarithmic scale; Ordinate: per cent of the control rate of respiration. Respiration measured with 10% KOH in the center well. T = 28.3°C.

When the affects of $HgCl_2$ and metallic mercury on endogenous respiration are observed in the presence of 10^{-2} M ethylenediamine tetraacetate (EDTA) in the cell suspension, then a marked difference between the two forms of mercury becomes evident. 10^{-2} M EDTA prevents 10^{-3} M $HgCl_2$ (in .05 M phosphate buffer of pH 6.5 and containing .01 M KCl)

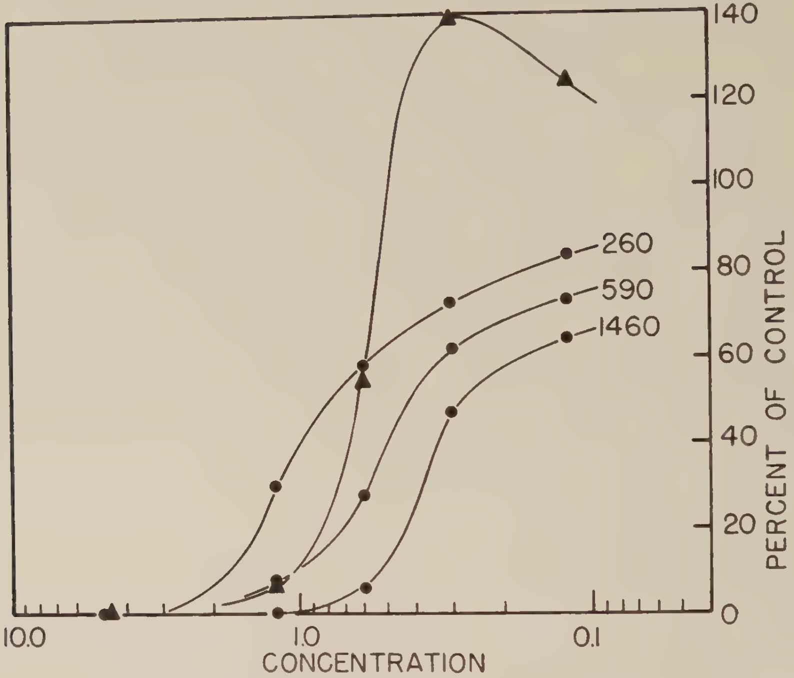

Fig. 3 Affect of ρ-chloromercuribenzoate on respiration and photosynthesis of *Scenedesmus* D_3. Suspending medium was Warburg no. 9 carbon dioxide buffer (Warburg, '19) (Na:K::65:35 and containing .01 M KCl). Abscissa: molarity of ρ-chloromercuribenzoate $\times 10^4$ on a logarithmic scale; Ordinate: per cent of the control rate. Filled triangles: respiration rates after 1300 minutes exposure to the poison; filled circles: photosynthetic rates after varying times of exposure to the poison, shown by the numbers (of minutes) alongside the curves. T = 28.3°C.

from exerting any affect on respiration, but, as is shown in figure 4, it does not influence the development of a respiratory stimulation in *Scenedesmus* D_3 from metallic mercury. If metallic mercury does indeed exert its toxicity as a mercuric ion or compound, then EDTA should also have been effective against poisoning by metallic mercury as long as the oxidation to the mercuric form occurred before the mercury passed into the cell. That it had no effect indicates that the oxidation occurred within the cell, from which EDTA was presumably excluded.

The possibility remains that the mercurous form is the toxically active substance in poisoning by metallic mercury.

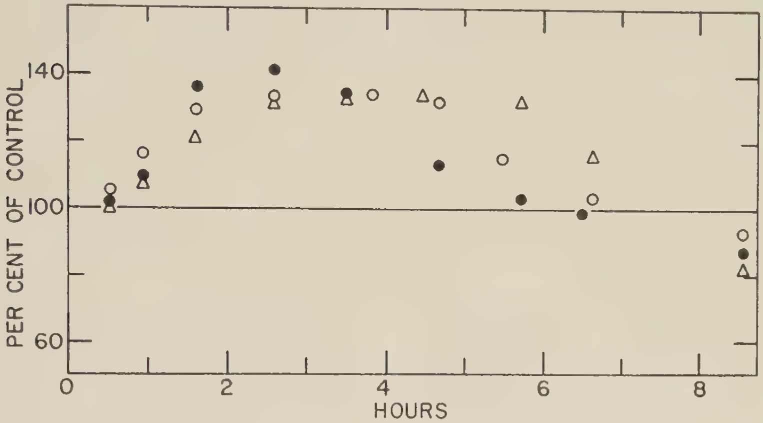

Fig. 4 The affect of EDTA and pH on the development of the mercury effect on respiration of *Scenedesmus* D_3. Abscissa: time in hours; Ordinate: per cent of the control rate. Filled circles: rates obtained with 1.5 gm of mercury in the Warburg vessel side arm, 10% KOH in the center well, and 32.5 µl of algae in 2 ml of .05 M phosphate buffer of pH 6.5 (containing .01 M KCl) in the main compartment, as compared to the appropriate control without mercury. Open circles: rates obtained under the same conditions except that 10^{-2} M EDTA is also present, as compared to the appropriate control without mercury. Triangles: rates obtained with 1.5 gm of mercury in the side arm and 30 µl of algae in 2 ml of Warburg no. 1 carbon dioxide buffer of pH 10.7 (Na:K::65:35 and containing .01 M KCl) in the main compartment, as compared to the appropriate control without mercury. T = 28.3°C.

In this case EDTA would, perhaps, not be expected to affect its toxic activity. However, thermodynamic considerations show that, if mercurous ion appears, then mercuric forms will appear in even higher concentration. In the presence of a metallic mercury phase, the equilibrium ratio of $Hg_2^{++}:Hg^{++}$ is 81 (Latimer, '38). In the absence of a mercury phase, as in a cell suspension exposed to mercury vapor in the air, it is reasonable that $Hg_2^{++} : Hg^{++} < 81$. Since mercurous salts seem to have little tendency to form complexes (Sherrill and Abegg, '03; Thorne and Roberts, '48), and there is much empirical justification for believing that "wherever (Complex mercurous compounds) might be anticipated the mercuric compound and metallic mercury are formed instead" (Barron, Flexner and Michaelis, '29), the total amount of mercurous forms will not differ much from the mercurous ion concentration. But the large tendency of mercuric ion to complex with chloride ion is given by Sherrill and Abegg ('03) as

$$\frac{(Hg^{++})\,(Cl^{-})^2}{(HgCl_2)} = 10^{-14}. \qquad (2)$$

When chloride ion is .01 M, the ratio of the mercurous ion, therefore, to mercuric chloride, the most abundant mercury species is $\frac{(Hg_2^{++})}{(HgCl_2)} < .81 \times 10^{-8}$. So even if some mercury were oxidized only as far as mercurous ion in the suspending medium outside the cell, EDTA ought still to have a very large affect on the development of the poisoning by metallic mercury.

Mercury, then, must enter the cell as unchanged metal atoms, where, in the presence of oxygen, intracellular substances stimulate its oxidation to a toxic mercury compound. Stimulation of the oxidation of metallic mercury by a variety of substances — organic and inorganic — has previously been described (Barron, Flexner and Michaelis, '29; Michaelis and Barron, '29), and explained by the partial reaction:

$$\text{(D)} \quad 2XA + Hg \rightleftharpoons HgX_2 + 2A^{+} + 2e.$$

XA is a substance that forms a complex with mercuric ion, and may be the thiol group of cysteine, a cyanide, a thiosulphate, or an iodide, but not a chloride. The rate of oxidation by molecular oxygen becomes larger as the affinity of XA for mercuric ion increases, which is reasonable since the reducing potential of the partial reaction involving mercury also increases and the free energy change in the reaction with molecular oxygen is larger.

It is a likely possibility that intracellular thiol groups are responsible for the oxidation of the mercury atoms, since thiol groups form strong complexes with mercuric ion and are of fundamental physiological importance (Barron, '51). Freedman and Corwin ('49) calculated an instability constant for mercuric cysteinate of only 5×10^{-23}. The great stability of mercury mercaptides explains not only the oxidation of uncharged mercury atoms but also the accumulation of mercury by cells and tissues exposed to metallic mercury (Stock, '34). The observation made by us and by previous workers (Dutton and Manning, '41) that the speed with which poisoning from metallic mercury develops is related inversely to the density of the cell suspension is certainly due to mercury accumulation.

Since the cell is not merely the passivle recipient of a dose of toxic oxidized mercury, when it is exposed to metallic mercury, but also is instrumental in causing the oxidation, there is not necessarily a strict parallelism between sensitivity to a mercuric compound like mercuric chloride and sensitivity to metallic mercury. In the absence of detailed information about the conditions necessary for oxidation of mercury inside the cell, it is not inconceivable that a cell may be very sensitive to mercuric chloride and still show little sensitivity to metallic mercury because it will not cause its oxidation. A corollary of this is that the metabolism of an organism need not be affected in the same way by an increasingly long exposure to metallic mercury as by increasing concentrations of mercuric chloride.

SUMMARY

1. *Scenedesmus obliquus,* strain D_3, is extremely sensitive to poisoning by metallic mercury. Mercury vapor diffusing over into a cell suspension from a side arm of a Warburg vessel can cause marked changes in metabolism to occur within less than an hour. Respiration can be stimulated up to 300% of the control rate while photosynthesis declines. Other effects are also observed.

2. A variety of other organisms, including green algae, a bacterium, a yeast, and leaf discs of higher plants also showed sensitivity to metallic mercury in varying degrees.

3. The poisoning of metabolism by metallic mercury is, in several respects, quite similar to the action of the uncoupling agent, dinitrophenol.

4. Evidence is advanced to support the contention that mercury in the mercuric form is the toxic agent and that the oxidation of uncharged mercury atoms to a mercuric compound occurs within the cell.

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THE REACTION TO HOT ATMOSPHERES OF VARIOUS SPECIES OF AUSTRALIAN MARSUPIAL AND PLACENTAL ANIMALS

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Physiology Department, University of Queensland, Brisbane

THREE FIGURES

Previous studies on the heat tolerance of Australian lower mammals (Martin, '03; Robinson, '54) have demonstrated the primitive type of heat regulation in monotremes (platypus and echidna) and the distinct advance in homeothermism shown by marsupials. Further experimental evidence of the comparatively high heat adaptability of marsupials is provided by the present study, which extends the available data from 6 to 30 species including a number which have become very rare. In addition to the 25 species of Australian marsupials, this investigation also compares the reactions to heat of 4 species of indigenous Australian rodents, a bat and a monkey.

METHODS

The animals were exposed in a psychrometric room for 6 hours to one or more of three selected hot atmospheres, with dry-bulb temperature of 35°C. (95°F.), 37.5°C. (99.5°F.), and 40°C. (104°F.). Absolute humidity was kept constant at 30 mm Hg. vapour pressure and air movement at an average velocity of 60 ft./min.

In some instances, when the number of individuals of a species was limited, the animal was re-exposed to the particular atmospheric conditions to confirm the previous obser-

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TABLE 1

Classification and body weights of the species

FAMILY	SPECIES	NAME	NO. INDIV.	BODY WT.
				gm
Dasyuridae	*Sminthopsis larapinta*	Stripe-head mars.-mouse	3	11
	S. crassicaudata	Fat-tail mars.-mouse	2	16
	Antechinus flavipes	Yellow-foot mars.-mouse	2	48
	Dasycercus cristicauda	Crest-tail mars.-rat	5	72 [1]
	Phascogale tapoatafa	Brush-tail mars.-rat	1	108
	Satanellus hallucatus	Little Northern nat.-cat	2	651
	Sarcophilus harrisii	Tasmanian devil	1	6,700
Peramelidae	*Isoodon obesulus*	Short-nose bandicoot	3	1,200
	Macrotis lagotis	Bilby or rabbit bandicoot	1	863 [1]
Phalangeridae	*Petaurus breviceps*	Sugar glider	1	117
	P. norfolcensis	Squirrel glider	2	236
	Schoinobates volans	Greater glider	1	1,070
	Trichosurus caninus	Short-ear brush-tail	1	3,190
	T. V. eburacensis	Cape York brush-tail	2	1,380
	T. V. vulpecula	Common brush-tail	4	1,950
	T. fuliginosus	Tasmanian brush-tail	1	5,060
Macropodidae	*Potorus tridactylus*	Long-nose rat-kanga.	2	1,010
	Setonix brachyurus	Short-tail pademelon	2	3,860
	Wallabia dorsalis	Black-stripe wallaby	2	2,790 [1]
	W. elegans	Pretty-face wallaby	1	8,750 [1]
	W. rufogrisea	Brush wallaby	1	9,700
	W. bicolor	Swamp wallaby	1	7,000 [1]
	W. agilis	Sandy or agile wallaby	1	18,740
	Macropus major	Grey kangaroo	1	30,700
	Megaleia rufa	Red kangaroo	1	30,800
Muridae	*Melomys littoralis*	Naked-tail rat	2	38
	Uromys sherrini	Giant naked-tail rat	2	508
	Hydromys chrysogaster	Water rat	2	686
	Rattus conatus	Field rat	1	90
Pteropodidae	*P. poliocephalus*	Grey-head fruit-bat	1	216
Cercopithecidae	*Macacus mulatta*	Macaque monkey	1	1,610 [1]

[1] Animals not fully grown.

vations. If body temperature reached a critical level, judged to be near the lethal limit, 40°C. (104°F.) for marsupials and 41.7°C. (107°F.) for placental mammals, before an exposure period of 6 hours had elapsed, the animal was withdrawn from the hot-room to cooler surroundings.

Animals were fed before but not during the experimental period; while free access to water was allowed during heat exposure.

The following observations were made immediately before the animals entered the hot-room (control readings) and thereafter at half hourly intervals (smaller species) or hourly intervals (larger species): rectal temperature — by thermocouple (smaller species) or by clinical thermometer (larger species); respiratory rate — by observing movements of the flank; general behaviour.

The species studied are listed in table 1. Nomenclature follows Troughton ('41).

RESULTS

Rectal temperature

Systematic studies on the variation of the normal body temperature in mammals has shown a strong correlation with activity both in American placental forms (Ryser and Morrison, '56) where increases of up to 2.5°C. may be seen, and in Australian marsupials (Morrison, '56), particularly in the *Dasyuridae* where rises of 4°C. are not uncommon. Therefore, control measurements taken before the animal entered the hot-room must be at an activity level comparable to that inside the hot-room if the values are to be properly compared. Accordingly, an animal was taken from its living quarters (often disturbed from sleep), it was weighed and, after its body temperature had been taken, was placed in a holding cage outside the hot-room for about 30 min. The "control" body temperature was then measured and the animal immediately transferred to the hot-room. These control temperatures are summarized in tables 2 and 3 and are in

TABLE 2

Respiratory rates and body temperatures at 35°C. and 37.5°C.

SPECIES	NO. OF TESTS	RESPIRATIONS PER MIN.				BODY TEMPERATURES IN °C.					
		At 35°C.		At 37.5°C.		At 35°C.			At 37.5°C.		
		Control	Exp.	Control	Exp.	Control	Experimental		Control	Experimental	
							Mean	Equilib.		Mean	Equilib.
D. cristicauda	4	135	80	102	117	37.3	36.9	36.8	37.6	38.8	38.9
S. hallucatus	1	176	151	108	132	35.9	37.3	37.2	35.8	38.1	38.0
I. obesulus	3	20	20	26	109	35.7	37.5	37.8	35.7	38.4	38.9
M. lagotis	1	20	23	30	40	34.8	36.7	36.8	34.6	36.9	37.9
T. v. vulpecula	4	60	114	55	133	36.9	38.4	38.5	36.5	38.6	38.6
T. caninus	1	56	82	65	86	36.9	38.5	38.3	36.8	38.7	38.8
P. tridactylus	1	100	85	52	139	36.5	37.6	37.6	37.3	38.9	38.9
M. littoralis	1			150	139				37.5	39.7	39.0
U. sherrini	1			200	144				37.7	40.7	41.3
H. chrysogaster	2	82	83	120	95	36.6	38.2	38.5	36.5	39.0	40.0
R. conatus	1	260	213	240	249	37.3	39.0	38.9	37.8	40.0	40.3

good agreement with mean values for the various species based on much larger series of data (Morrison, '56). Figures show a fairly close agreement in body temperature amongst the different species of marsupials with the noticeable exception of the bilby. The very low values observed in this animal are a result of its extremely phlegmatic temperament. Control rectal temperatures of rodents fell within the range of variation of values for marsupials, that of the monkey was higher, and that of the fruit-bat was lower.

When exposed to heat, the rectal temperatures of all species rose during the first two hours. Then, if the temperature conditions were not too severe, a new equilibrium between heat gain and heat loss was reached. Tables 2 and 3 show the body temperatures at which this thermal equilibrium was established for different species and gives some indication of their stability. Body temperatures of the small marsupials rose so rapidly at first that they tended to overshoot this stable value owing apparently to a time lag in amplifying the heat dissipating mechanisms. Later a noticeable fall to the steady state was observed. Differences in the rate of rise of body temperature of small and large marsupials during the first two hours of heat exposure are illustrated in figure 1.

Maintenance of body temperature at a constant but generally higher than normal value was successfully achieved by all the species at an air temperature of 35°C. However, when the ambient temperature was raised to 37.5°C. a somewhat unstable equilibrium was noted in the bilby, and giant naked-tail rat.

More severe heating (40°C.) produced noticeable generic differences in body temperature reactions, the small carnivorous species being particularly intolerant. A steady state was achieved either only temporarily or not at all by the 5 species of marsupial-mice and -rats, the bilby, and by 2 rodents — the naked-tail rat and water rat. Average durations before the animals had to be removed with a critical temperature ranged from 1.5 hr. in the giant naked-tail rat to 5.3 hr. in the marsupial-mouse. When water was denied the marsupial-

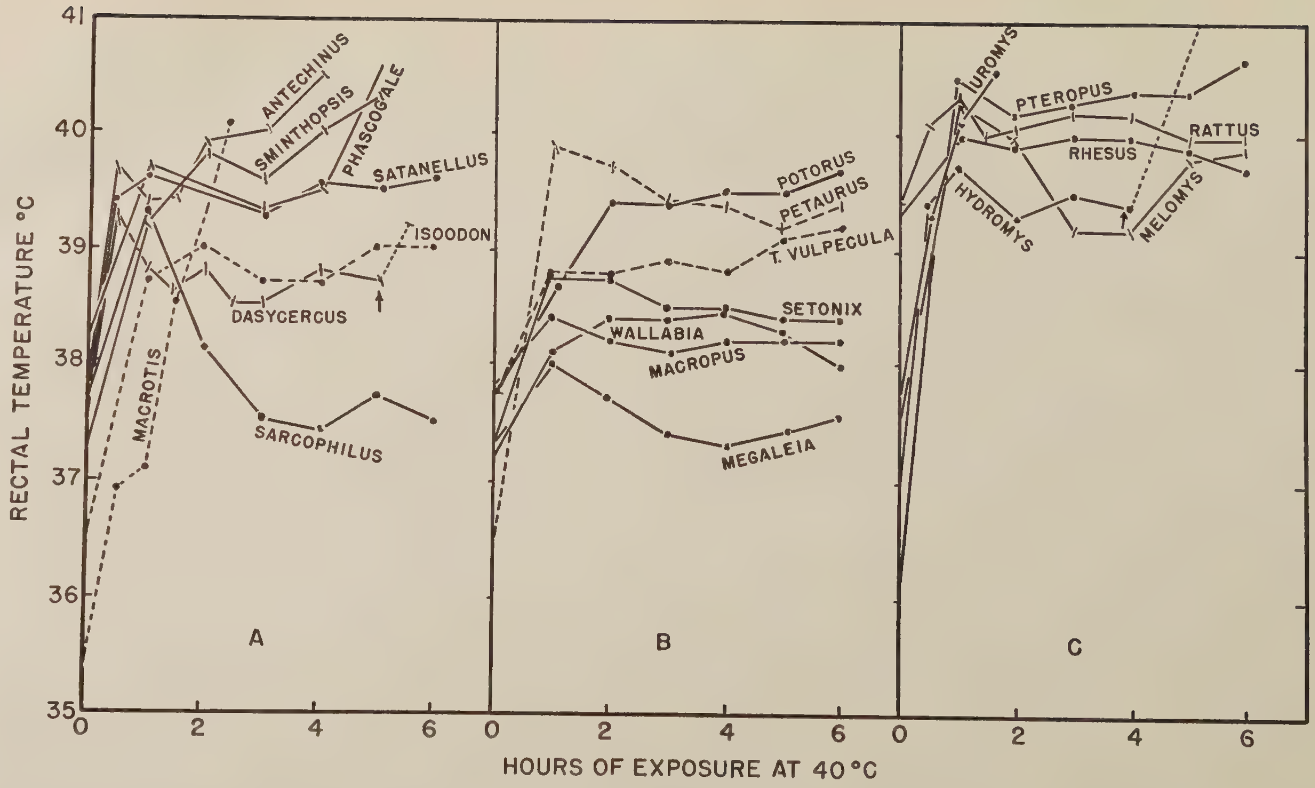

Fig. 1 Effect of a hot atmosphere (40°C., 30 min. Hg. v.p.) on the rectal temperature of various mammals: (A) Carnivorous marsupials; solid curves, dasyuridae; broken curves, peramelidae; (B) Hervivorous marsupials; broken curves, phalangeridae; solid curves, macropodidae; (C) Rodents, monkey and fruit bat. Bars indicate small animals (< 100 g). Values are means of several individuals of a species. Arrows indicate withdrawal of drinking water.

mice or water rat during heat exposure, the rise in body temperature was precipitous (fig. 1 A and C). Of the different marsupial-mice, the yellow-footed species showed the greatest intolerance to heat. Although these body temperatures are fairly heat-labile in small dasyurids, it should be noted that they tolerated a higher body temperature than was previously observed in macropods and phalangers (Robinson and Lee, '46).

All 7 species of phalangers achieved thermal equilibrium at an air temperature of 40°C. There was little variation between these animals in their rectal temperature response and their heat tolerance was comparatively high. A mean body temperature, somewhat below the range of values for the other species, was noted in the greater glider. This difference might be related to a greater heat loss from the very large gliding surface area, but that the body temperature was lower than the ambient temperature of 40°C. and these gliding surfaces were not exposed. A more probable explanation involves the excellent insulating layer — a fine, long fur — and a reduced metabolic rate in this very gentle species (Morrison, Harvey and Morris, '56). Among the macropods, the small rat-kangaroo showed more heat strain than the possum under the same conditions of heating. However, the wallabies exhibited a low rectal temperature response to hot atmospheres with a high degree of uniformity between species, with the single exception of the black-stripe wallaby. The latter data is in some question since these two individuals were the most immature of the group and were very excitable. Heat tolerance of the two kangaroos either equalled or bettered that of the wallabies.

Tables 2 and 3 illustrate the improved heat regulation of the phalangers when compared to the carnivorous marsupials. It will be seen that a rise in air temperature from 35° to 40°C. produced little change in mean body temperature of both species of possum during a 6 hour exposure while rises in the other genera were much greater. The rise was maximal in the marsupial-mice.

TABLE 3

Respiratory rates and body temperatures at 40°C.

SPECIES	NO. OF TESTS	RESPIRATION PER MIN.		BODY TEMPERATURE IN °C.			STABILITY OF EQUILIB.
					Experimental		
		Contr.	Exp.	Contr.	Mean [1]	Equilib.	
S. larapinta	3	176	179	37.2	(39.5) *5.3*	(40.0)	Poor
S. crassicaudata	2	205	260	38.7	(39.2) *5.3*	(39.5)	Poor
A. flavipes	2	156	178	37.6	(40.0) *4.0*	(40.0)	Poor
D. cristicauda	5	152	206	37.3	(38.8) *5.0*	(39.2)	Poor
P. tapoatafa	1	180	171	38.1	(39.6) *5.0*	(39.5)	Poor
S. hallucatus	2	176	225	37.3	39.2	39.5	Good
S. harrisii	1	116	127	37.6	37.9	37.5	Good
I. obesulus	3	58	131	36.2	38.6	39.1	Fair
M. lagotis	1	24	58	35.3	(41.6) *2.0*	. . .	None
P. breviceps	3	187	180	36.9	38.4	38.5	Good
P. norfolcensis	2	120	150	36.3	39.3	39.5	Good
S. volans	1	80	121	36.5	38.0	38.1	Fair
T. V. vulpecula	4	78	171	37.0	38.8	38.9	Good
T. V. eburacensis	2	122	148	36.3	38.5	38.7	Fair
T. caninus	1	56	122	36.9	38.6	39.0	Good
T. fuliginosus	1	120	164	36.2	38.5	39.1	Good
P. tridactylus	2	104	144	37.7	39.2	39.5	Good
S. brachyurus	4	110	195	37.3	38.4	38.5	Good
W. dorsalis	2	110	200	37.4	39.8	39.9	Good
W. elegans	1	44	168	36.7	37.9	38.1	Good
W. rufogrisea	1	120	197	37.7	38.8	38.8	Good
W. bicolor	1	96	206	39.3	38.3	38.0	Good
W. agilis	1	106	185	37.9	38.0	37.9	Good
M. major	2	60	158	37.2	38.2	38.4	Good
M. rufa	2	92	204	36.5	37.0	37.2	Good
M. littoralis	2	170	176	37.5	(39.4) *5.2*	39.5	Good
U. sherrini	2	190	241	37.1	(40.4) *1.5*	. . .	None
H. chrysogaster	2	112	96	36.9	(39.6) *5.2*	(39.9)	Poor
R. connatus	2	220	199	39.0	40.0	40.1	Good
P. poliocephalus	1	68	202	36.2	40.0	40.4	Good
M. rhesus	1	76	85	39.3	39.9	40.0	Good

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[1] Figures in parentheses have been weighted because of removal of the animal before 6 hours; italicized figure indicates average hours in the heat.

Respiratory rate

Normal respiratory rates at rest in the absence of heat stress are given also in tables 2 and 3. These single values are of course less reliable than the mean of the 6 values during heat exposure, particularly since heat has a sedating effect. Slow rates (20–30/min.) were shown by the bandicoot and bilby; moderate rates (60–120/min.) by the Tasmanian-devil, phalangers (except the small sugar-glider), rat-kangaroo, wallabies, kangaroos, water-rat, fruit-bat, and monkey; and rapid rates (> 150/min.) by all the small species: marsupial-mice and rats, native-cat, sugar-glider, naked-tailed rats, and bush rat.

Most species showed increased respiratory rates under hot conditions, noticeable exceptions being the Tasmanian-devil, bilby, sugar-glider, naked-tailed rat, water rat, field rat, and monkey (tables 2 and 3; fig. 2).

Behaviour

Certain behavioural patterns common to all homeotherms when exposed to hot atmospheres (Robinson and Lee, '46) were again evident in this investigation:

(i) drowsiness and general decrease in activity to minimize heat load;
(ii) postural changes to increase body surface for heat disposal;
(iii) vasodilation on all sparsely haired areas, e.g., ears, feet, tail;
(iv) increase in evaporative cooling of the body either by sweating, panting, or wetting the fur by licking or immersion in any water available.

However, there were noticeable differences amongst the genera in the degree to which these patterns were employed, particularly that of evaporative cooling.

All species of marsupial-mice limited their coat licking to the front and hind paws and base of the tail preferring rather to cool themselves by sitting in or on their water tins. No

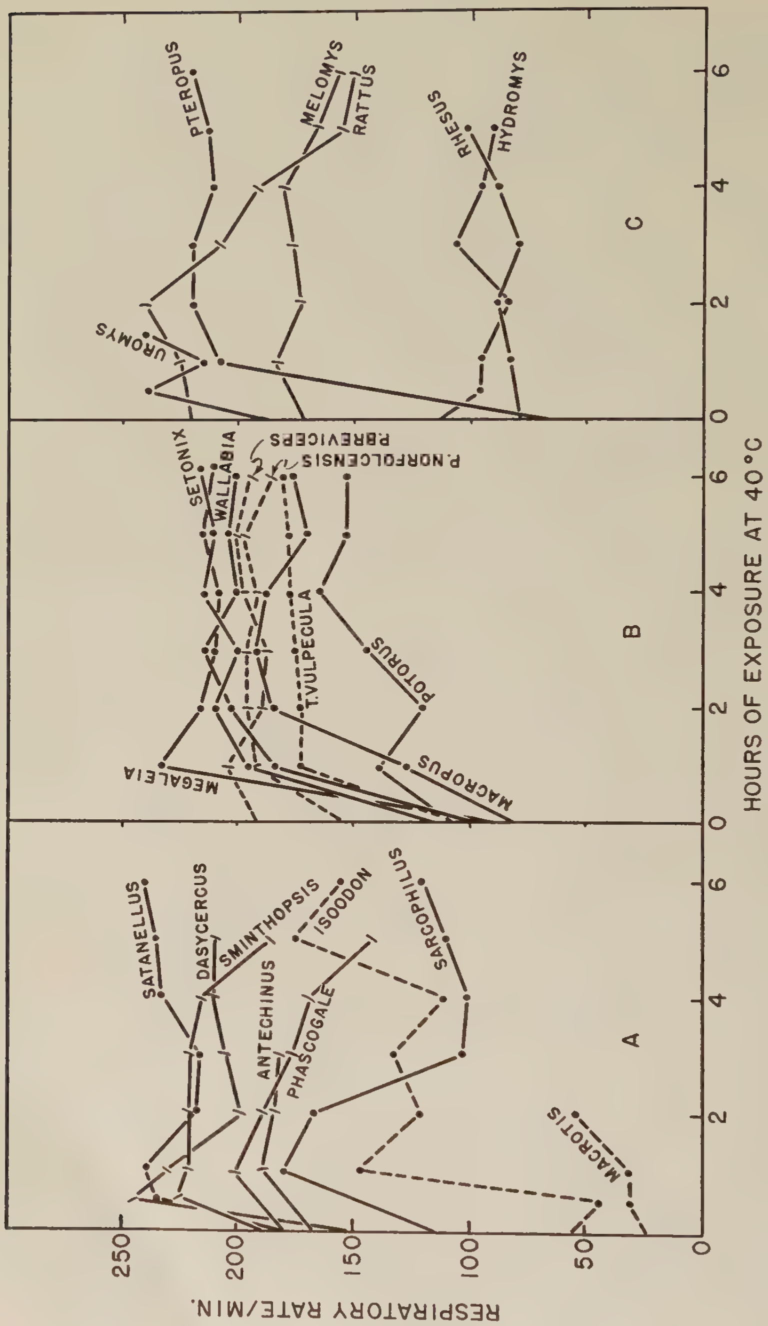

Fig. 2 Effect of a hot atmosphere (40°C., 30 min. Hg. v.p.) on the respiratory rate in various mammals. Symbols as in figure 1.

attempt was made to drink during heat exposure. The mouth was opened slightly with panting and, when hot, the animal usually adopted a lizard-like posture.

The marsupial-rat and native-cat both replaced rapid breathing by open-mouthed panting at body temperatures of 39°C. The coat-licking extended to the abdominal fur with the marsupial-rat but was restricted to the fore and hind paws in the native-cat. Of these two species, only the native-cat was seen to lap water during heat exposure.

The only observable reaction of the Tasmanian-devil to an air temperature of 40°C. was its high water consumption. Throughout the period of exposure, it showed no distress. There was no change in respiratory rate and no attempt was made to wet the coat.

When body temperature of the bandicoot approached 39°C. at a dry-bulb of 40°C., it commenced licking its forepaws and abdomen. This had a considerable cooling effect, skin temperature in the wetted parts showing a drop to 36.1°C. The animal lay stretched out on its side when hot and panting with mouth open began after a small rise in body temperature (38.5°C.).

Vasodilation of the large ears, mouth parts and paws was quite marked in the bilby. The animal lay at first stretched out on its side, but at a body temperature of 38.5°C. (a rise of 3°C. above the initial value) it became restless and at 39.8°C. was in obvious distress. When transferred to cooler surroundings (25°C.), its body temperature fell 3°C. in 30 mins. No attempt was made by the bilby to increase evaporative cooling by any of the possible methods and no water was drunk during heat exposure.

Coat-licking in all species of possum was more extensive than in any of the above species. With lower body temperatures, it was confined to fore and hind limbs but this was later extended to include the abdomen, scrotal region and undersurface of the tail. The animal then assumed a sitting posture to expose the wetted areas. Open-mouthed panting was employed and small quantities of water were repeatedly drunk.

A noticeable feature of the behaviour common to the rat-kangaroo (in a minor degree) and to all species of wallabies and kangaroos in hot atmospheres was the profuse licking of the fore and hind limbs, open-mouthed panting, and copious drinking. After 3¾ hours exposure to an air temperature of 45°C., heat staggers, followed later by death, developed in the quokka (short-tail pademelon) at body temperature of 40.9°C. (105.6°F.).

Of the 4 species of indigenous Australian rodents only the giant, naked-tail rat made any attempt to increase evaporation by licking of coat, the three other species preferring to use their drinking water to wet the fur. This behaviour was particularly marked in the water rat, an animal which has become remarkably adapted for aquatic life. As its rectal temperature approached 39.5°C., it endeavoured to immerse as much of its body as possible, finally resting with its head in its water tin. At higher body temperature (41°C), it lay on its back with moist abdomen exposed. No attempt was made to increase respiratory activity and at a body temperature of 43.5°C. (110.3°F.) death due to heat stroke occurred. Of the rodents, drinking was observed only in the water-rat.

The bat hung quietly with head downwards until its body temperature rose to 38.5°C. Then it began licking its forearms, panting with open-mouth and showing great activity with flapping of wings. The wing-fanning was a marked feature of its reaction to heat and continued ceaselessly for the remaining 5 hours.

DISCUSSION

An immediate response of all animals to hot atmospheres was a rise in rectal temperature. However, at air temperature of 40°C., thermal equilibrium was established at a higher level in all but the most anatomically primitive species of marsupials and in some of the indigenous rodents. Differences between animals in the degree of rise above initial body temperature at which a steady state was achieved appeared to be inversely related to the animal's ability to intensify evaporative cooling.

Results indicate that differences of tolerance among the species in the first hour to two of heat exposure are related in part to body size. The smaller species accumulated heat in the body much faster than the larger. This relates to their higher metabolic intensity which accentuates any thermal unbalance, and also to their higher surface, mass ratio and thinner insulation which both facilitate heat exchange with the environment.

Among the dasyurids, the 3 marsupial-mice, the 2 marsupial-rats and the native-cat are quite similar in form, habit, and in the hot, dry environmental conditions to which they are usually exposed for long periods during the year, but they represent a size series ranging some 60-fold (11–650 gm) and therefore might react differently to the same heat stress. However, they show a quite uniform response to heat with stabilized body temperatures, under 40°C. heat stress, ranging from 39.2–40.0°C. for the 5 species. It must be emphasized that this often represented only a transient stabilization and longer exposure resulted in a higher, unstable body temperature.

A rise in rectal temperature during heating, particularly in those animals with poor heat dissipating mechanisms, is protective, in a sense, since with the higher skin temperature which follows raised body temperature, less heat will be absorbed from the surroundings. In addition, at air temperatures below that of the skin, the important avenues of radiation and conduction for heat dispersal would be available to the animal.

These non-evaporative methods for heat loss would appear to be particularly well developed in the bilby, an animal in which vasomotor responses to heat are quite marked, particularly in the long, bare ears. However, opportunities for heat loss by these avenues are only possible when air temperature lies below skin temperature; and this would explain both the tolerance of the bilby to moderately hot atmospheres and its intolerance to very hot conditions. Field and laboratory observations and body temperature measurements on this animal show a very strongly developed 24-hour cycle with the active phase at night (Morrison, '56). Under ordinary circumstances, therefore, the bilby would utilize the cooler microclimate of its deep burrow and thus not be subjected to radiation or high ambient temperatures. It is of interest that the other paramelids, the long-nose and short-nose bandicoots, which show a limited use of salivation for evaporative cooling, are not adapted to dig a protective burrow.

The thick furry covering of mammals in general, which is an important insulator against cold, would also protect the animal from excessive radiation and conduction from hot atmospheres and thus reduce the effective heat stress under a given climatic condition. It is particularly well developed in the phalangers and is undoubtedly an important factor in the moderately high heat adaptability of these families.

The four brush-tail possums present a good series comparing closely related animals of this group which encounter different environmental conditions. They come respectively from Cape York in the tropical north *(T.v. eburacensis)*, from coastal, south Queensland *(T.v. vulpecula)*, from the somewhat cooler, moister mountains in south Queensland *(T. caninus)*, and from Tasmania south of Australia proper *(T. fuliginosus)*, thus representing a north-south range of about 30° in latitude or more than 2,000 miles. These animals show a regular progression in size (1.4–5.1 kg) from North to South, as listed, and the latter two species are definitely of more compact, stocky build than the former two and have if anything a longer fur. However, although a slight trend in regulation was observed in this group (fig. 3), it cannot be considered significant and indeed variation between these species was no more than between some individual animals.

In the larger forms with good insulation, the heat load might be sufficiently low that a tolerance to rising body temperature could be effective in conserving water which otherwise must be used for evaporative cooling. The Schmidt-Nielsons ('55) have recently shown that such heat buffering by the body with temperature increases of as much as 6°C. is a very important factor in the survival of the camel under hot, dry conditions in the Sahara. The hot, dry conditions encountered in Central Australia appear such as to make this a useful adaptation in some kangaroos and wallabies. However, of all the marsupials these have the most uniform body temperatures, often varying by less than 0.3°C. from day to night. And during heating experiments they showed the least temperature change, expending salivary water freely to keep the body temperature at a relatively low level. However, these animals were not subject to water deprivation, and camels given free access to water maintain a much more uniform body temperature through sweating.

Although there is much histological evidence to indicate that the apocrine type of ''sweat gland'' is universally distributed throughout the marsupials (Hardy, '47), the use these animals make of saliva to wet their fur would suggest that the thermoregulatory function of these glands is not of a high order. From our own observations on the macropod, *Setonix,* salivary application rather than activity of sweat glands would seem a more probable explanation for the ''copious sweating from the fore and hind paws'' described by Bently ('55), during heat exposure of this species. This was also the conclusion of Bartholomew ('56) who made a careful study of *Setonix.* Moreover, tests (Higgenbotham and Koon, '55) for thermal sweating on the Virginia opossum by the starch-iodine technique failed to demonstrate sweat gland activity in any region of the body surface. Thus, while histological evidence alone might suggest that the presence of sweat glands provides marsupials with an advantage over rodents in hot atmospheres, when viewed in the light of functional evidence this seeming advantage disappears. In any event it is questionable whether the release of sweat at the skin surface in forms with such good insulating layer, as the phalangers and macropods, would result in effective evaporative cooling.

A notable exception in response was the Tasmanian-devil which was able to maintain thermal equilibrium at only a little above normal body temperature. Since this animal makes no attempt to intensify evaporation from the respiratory tract or from body surface by wetting the fur, the only possible explanation for its high heat tolerance, when air temperature exceeds body temperature, would seem to be an effective sweating mechanism. Its sparsely haired coat would afford it little protection from the hot atmosphere but at the same time would give minimal interference with the evaporation of sweat. Although sweat was never observed running from the animal, its skin was definitely damp.

While a close agreement in powers of heat regulation and in the methods adopted by the various species within a family were evident from this study, differences between families in respect to both these factors are apparent.

The marsupial-mice and -rats make no attempt to increase body evaporation although they may use other available water for cooling. Together with the bandicoot, and bilby, they rely mainly on radiation and conduction for heat dispersal. When the air temperature rises above the body temperature, these animals become intolerant to heat. The better heat adaptability of the more highly evolved family, the phalangers may be attributed to two factors (a) the thick coat which provides these animals with protection against much heat absorption from hot atmospheres and (b) an improved evaporative cooling mechanism of respiratory and salivary origin. An intensification of evaporative cooling through open-mouthed panting and profuse licking of fore and hind limbs together with the very effective insulating value of their fur would account for the even higher heat tolerance of the macropods — wallaby and kangaroo. Of this family, the smallest and most primitive member, the rat-kangaroo, relied least on evaporative cooling and its reaction to heat was greater.

Differences in water consumption between species during heat exposure paralleled the degree of evaporative cooling — substantial in macropods, moderate in phalangers, and negligible in the others thus ensuring the continuence of this thermoregulatory mechanism.

In brief then, the degree of development in marsupials of an evaporative mechanism for heat removal, whether respiratory or by coat-licking, differs between species and parallels structural evolutionary trends. It is highest in the macropods, somewhat less in the phalangers, still less among the bandicoots and minimal in the desyurids, marsupial-mice and -rats, the family considered most nearly to approach the primitive marsupial stock.

However, this rather satisfying series in which morphological and physiological specializations are so nicely correlated may give the impression that the "primitive" dasyurid marsupials are a rather helpless, ineffectual group, perhaps comparable to the Xenarthra among the higher mammals. Actually, they are the most intelligent and in many ways effective of the marsupials, and are well adapted for survival in the hot, dry areas to which they are often indigenous. The important factor here is that of size. The marsupial mice and rats at one end of the scale being only one hundreth the size of the macropods at the other end, with the peramelids and phalangers between. This factor acts first, physiologically, by increasing the heat stress imposed on a small animal by a given condition and second, ecologically, by presenting the opportunity of utilizing the cooler micro-environments underground and thus, by evasion, obviating the need for active heat regulation. Among the dasyurids, the outstanding exception was the Tasmanian Devil which showed a highly developed capacity for heat regulation, fully as good as that seen in any of the macropods — but it is also the one member of this group which is in the larger size class. Of the other groups, the kangaroos and wallabies have little chance to avoid heat stress in the open areas where they are usually found, and the phalangers, except for the limited rain forest

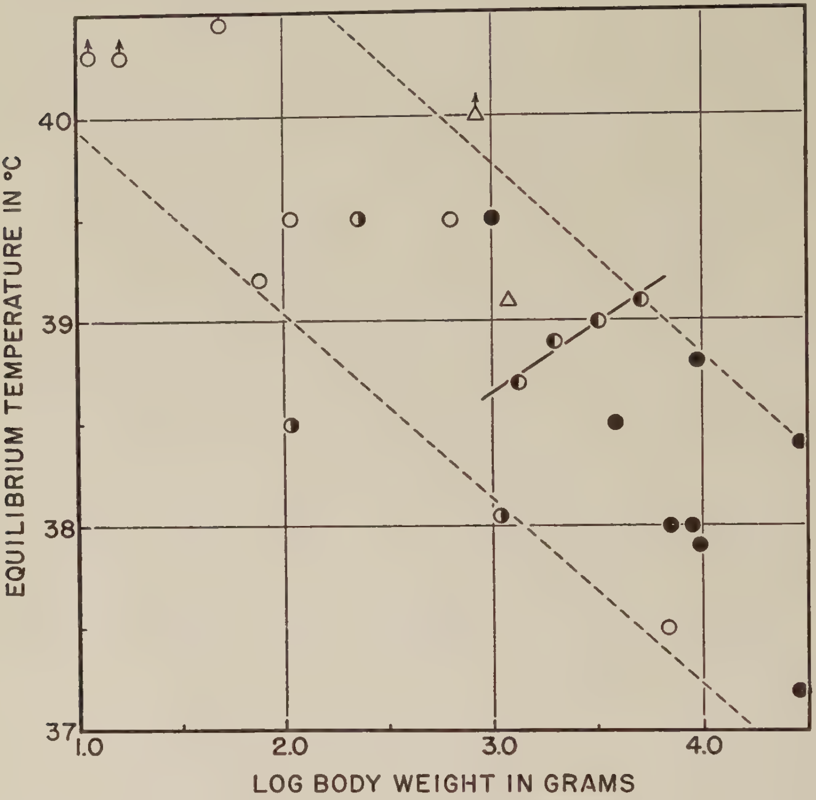

Fig. 3 Relation between the increase in body temperature at 40°C., 30 min. Hg. v.p. and the body weight. Open circles, dasyuridae; triangles, peramelidae; half-closed circles, phalangeridae; closed circles, macropodidae. Mean values for species. Solid curve indicates trend in values for 4 species of *Trichosurus*. Broken curves show approximate limits of the data.

area, are also largely exposed to the macro-climate since the thinly-leaved, eucalyptus flora affords little protection from heat. However, by their larger size they are spared in part the effects of this warmer microclimate. These relations are summarized in figure 3.

Rodents are well known to be poor reactors to hot atmospheres (Stigler, '30; Lee and Robinson, '41). And the indigenous Australian members of this Order studied here proved to be no exceptions. Their intolerance to heat may be explained by their failure to employ evaporative cooling methods to any extent and, of course, their small size. Of the accessory methods of evaporation, wetting of fur from external sources was that most favoured. If a supply of water for this purpose was unavailable, body temperature rose rapidly to lethal levels.

In the fruit-bat, the fanning response would be effective through conduction and convention at ambient temperatures below body temperature. This concept is supported by the findings of Reeder and Cowles ('51) that bats can regulate their body temperature by controlling the circulation in the wings, in which the available area for heat loss is very large. However, at 40°C these avenues for heat dispersal no longer exist. Possibly this response pattern is related to lower ambient temperatures, and there is no provision for stopping it at higher temperatures which ordinarily would not be encountered in nature. An alternative explanation is that the mechanism which governs the synchronization of respiration with wing beats in bats during flight (Krogh, '41) might also operate under a heat stimulus. Fanning would hardly be necessary for evaporative loss from the wings, and the extra work involved would add to the heat load.

Hardy's observation ('53) that the monkey, when exposed to an air temperature of 42°C. had a skin temperature 3°C below body temperature of 40°C., strongly suggest that the sweat glands of this animal were actively secreting. It would also explain our finding of a high heat tolerance in the monkey whilst showing none of the expected physiological adjustments of animals to heat. The Tasmanian-devil is the only other animal in this investigation in which sweat gland activity appears to be of importance in thermoregulation.

In table 4, the response of body temperature to hot atmospheres of animals of this series is compared with that of

TABLE 4

Comparison of the heat-regulatory ability of mammals at 40°C.

GROUP	THERMAL EQUILIBRIUM			
	None	Δ T > 2°C.	2°C. > Δ T > 1°C.	Δ T < 1°C.
Monotremata	Echidna[1] Platypus[1]			
Dasyuridae	*Mars. Mice* *Mars. Rat*	*Nat. Cat.*		*Tas. Devil*
Peramelidae	*Bilby*	*Bandicoot*		
Phalangeridae			*Gliders* *Possums* Cuscus[1]	
Phascolarctidae			Koala[1]	
Macropodidae			*Rat. Kanga.* *Pademelon*[10] *Wallabies*	Wallaroo[1] *Kangaroos*
Rodentia	*Giant naked-tail rat* *Water rat* Rabbit[2]	*Naked-tail rat* White mouse[3] White rat[3]		*Field rat*
Chiroptera		*Fruit bat*		
Carnivora		Cat[4]		Dog[5]
Artiodactyla	Pig[6]		Cow[7]	Sheep[8]
Primata				*Monkey* Man[9]

Current animals in italics; others as follows:

[1] Robinson ('54).

[2] Lee and Robinson ('41a).

[3] Robinson ('55).

[4] Robinson and Lee ('41a).

[5] Robinson and Lee ('41b).

[6] Robinson and Lee ('41c).

[7] Robinson and Klemm ('51).

[8] Lee and Robinson ('41b).

[9] Lee ('40).

[10] Bartholomew ('56).

other mammals from earlier studies, in which the experimental procedure was similar. Animals are grouped according to: (i) their ability to establish thermal equilibrium during a 6 hour exposure to air temperature of 40°C. and (ii) the elevation of this stabilized level above normal body temperature. The more primitive species tend to be grouped towards the left of the table and those most highly evolved structurally (with the exception of the pig), to the right. Of interest is the remarkably high heat tolerance shown by the Tasmanian-devil.

TABLE 5

Comparison of respiratory response in mammals at 40°C.

NORMAL RATE	MODIFICATION AT 40°C.	
	Slight	Marked
Slow	Echidna[1]	*Bandicoot*
(20–30/min.)	Platypus[1]	
	Bilby	
	Man[9]	
Moderate		*Gliders*
(60–120/min.)	*Tas. Devil*	*Possums*
	Cuscus[1]	Koala[1]
	Water rat	*Wallabies*
	White rat[3]	Wallaroo[1]
	Monkey	*Kangaroos*
		Rabbit[2]
		Fruit bat
		Cat[4]
		Dog[5]
		Pig[6]
		Sheep[8]
		Cow[7]
Fast	*Sugar glider*	*Mars. mice*
(> 150/min.)	White mouse[3]	*Mars. rat*
	Naked-tail rat	*Nat. cat*
	Field rat	
	Mars. rat	

The current animals in italics; literature references as in table 4.

The respiratory reaction to hot atmospheres of various mammals is summarized in table 5. Respiratory activity constitutes a most important method of heat regulation for animals which do not sweat readily. Therefore, those species which do not employ it must either rely on some other avenue for heat disposal or fare badly.

We may sum the findings of this inquiry in 4 statements. First, temperature regulation of marsupials, generally, compares well with that of many placental mammals; indeed, the phalangers and macropods are less stressed by hot conditions than are the poorly heat adapted rodents. Second, powers of heat regulation and methods adopted generally show close agreement by the various species within a family but noticeable differences between families. Third, improvement in the capacity for evaporative cooling is largely involved and is associated with evolutionary structural specialization. Finally, this correlation of heat-resistance with taxonomy, or degree of structural evolution appears in large part due to accompanying differences in size and behavior of the animals studied which at once reduce the effective environmental heat stress and increase the physiological heat sensitivity in the smaller, more primitive group.

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SUMMARY

Observations were made on the reactions to hot atmospheres of 25 species of Australian marsupials, 4 species of indigenous Australian rodents, a fruit-bat and a monkey. Heat tolerance of marsupials compared well with rodents and the bat, but was inferior to that of the monkey. It varied noticeably between species showing structural evolutionary trends. Improvement in thermoregulation was associated with greater reliance on evaporative cooling. These findings are in keeping with those of previous studies on temperature regulation in marsupials.

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A COMPARATIVE STUDY OF ERYTHROCYTE METABOLISM[1]

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NINE FIGURES

It has been known for many years (Warburg, '09; Ramsey and Warren, '30, '34; Michaelis and Salomon, '30) that the nucleated erythrocytes of birds consume oxygen at a much higher rate than the non-nucleated erythrocytes of mammals. A number of investigators including Laskowski ('42), Dounce and Lan ('43) and Hunter and Banfield ('43) attempted to find a basis for this difference by measuring the oxidative activity of isolated nuclei. The respiratory activity of these nuclei, however, was not appreciably higher than that of whole cells. The difference in oxygen consumption might be due to one of the following factors: (1) Quantity of specific enzymes available; (2) Quantity of specific substrates available; (3) Quantity of cofactors or inhibitors present; or (4) Local differences in pH, temperature, etc. The present investigation is an attempt to test the first of these 4 possibilities.

Such a study takes on added significance in view of the present interest in the mechanism involved in cation exchanges in erythrocytes. Several groups of workers including Weller and Taylor ('51), Solomon ('52), Maizels ('51) and others have suggested that metabolic energy is involved in maintaining the imbalance of Na and K between erythrocytes and their environment. Enzyme studies might indicate the energy source for this process. Also, from the viewpoint of comparative physiology and biochemical evolution it would be valuable to have information about the enzymic constitution and general metabolism of these highly specialized and important cells.

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[1] This work was performed in the Physiol. Dept., Florida State U. and was aided by a contract with the Office of Naval Research, NR 120-077.

Before undertaking this investigation it was necessary to determine whether the generalization could be made that erythrocytes of all the lower vertebrates respire at a level higher than that of mammalian red cells. It is difficult to make comparisons using the values in the literature since the measurements of the activities of fish, frog, turtle and snake bloods were made at 20° or 25°C., while the measurements with avian and mammalian bloods were made at 37–38°C. (Albritton, '51).

The present investigation consists of two parts: (1) measurements of the respiration of erythrocytes of 6 different vertebrates at 20°C. and 37°C., and (2) assays of the activity of several enzymes in these cells. Enzymes which might be responsible for differences in respiration such as succinoxidase and malic oxidase from the aerobic Krebs cycle were studied. As regards glycolysis, the nucleated avian erythrocytes show negligible aerobic lactic acid production but appreciable anaerobic glycolysis (Negelein, '25), while non-nucleated cells show a high glycolytic rate both aerobically and anaerobically (Bird, '47). From glycolysis, the terminal enzyme, lactic dehydrogenase was chosen as a convenient one to study.

MATERIALS AND METHODS

The blood from humans was obtained by venipuncture and that from rabbits, chickens, frogs *(Rana pipiens)* and sting ray *(Dasyatis)* by cardiac puncture. Fish *(Haemulon plumieri,* white grunt) blood was collected by cutting the branchial arteries which may have resulted in some contamination by body fluids and sea water. Heparin was a satisfactory anticoagulant in all cases except for fish blood which frequently clotted during collection. The clots redissolved, however, before the blood was used. Defibrinated and heparinized bloods were compared and similar results were obtained, indicating that heparin did not affect the enzyme activity.

In some experiments the whole ray blood was stored on ice for about 2 hours, and the fish blood for 3–4 hours since the laboratory is some distance from the coast.

The whole blood was centrifuged at 2000 × g. for 10 minutes and the plasma and buffy layer were removed by aspiration. The erythrocytes were washed in isotonic saline or Ringer-Locke solution twice. They were immediately frozen in dry ice, and thawed at 37°C. This was repeated twice and the resulting hemolysate made up to the approximate dilution desired with phosphate buffer or water. It was frequently necessary to use a glass Ten-Broeck homogenizer in order to disperse the ghosts through this mixture.

Respiration measurements and enzyme assays were made at 37°C. in a standard Warburg apparatus. In general, assays previously described in the literature were used. To determine optimum concentration of substrates and added cofactors, the linearity between activity and homogenate (enzyme) dilution was tested for each system.

Routine C. P. chemicals were used except for DPN, which was purchased from Schwartz Co., and cytochrome c from Sigma Chemical Co. The purity of these latter two materials was tested spectrophotometrically. All the solutions used were adjusted to pH 7.4 with KOH or HCl.

RESULTS

Respiration of whole cells

The oxygen consumption of whole washed red blood cells was determined at 20°C. and 37°C. In some experiments the respiration of 2–3 samples of the same blood was determined for 60–90 minutes at 20°C. and then at 37°C. following a 30 minute interval for temperature adjustment. In other experiments, simultaneous measurements of oxygen consumption in two different baths at these temperatures were made. The blood was equilibrated with oxygen before measuring the respiration to insure oxygenation of the hemoglobin. The data are shown in table 1. Here, as in all the following tables, the Q_{O_2} refers to the microliters of oxygen consumed per milligram of dry weight per hour. The weight was determined on 0.7–1.0 ml duplicate samples dried to constant weight at approximately 100°C.

In general the respiration data are comparable to those previously reported. The mean for chicken cells is lower than that in the literature, but additional data collected during other experiments in this laboratory give a mean of 0.142 and indicate that this is a reliable figure. As was expected, the oxygen consumption of the nucleated erythrocytes is significantly higher than that of the non-nucleated ones when compared at the same temperature. A possible explanation for the high respiratory rate of the sting-ray erythrocytes might be found in the work of Dawson ('33), who demonstrated that elasmobranch blood has a large number of immature red cells. In mammalian blood, at least, these immature cells consume oxygen at a much higher rate than do mature erythrocytes. As Ponder ('48) points out one must keep in mind the large quantity of inert hemoglobin present in red blood cells and when a correction is made for this by multiplying the values obtained by a factor of at least 6, erythrocytes have oxidative activity comparable with other tissues.

TABLE 1

Oxygen consumption of whole erythrocytes

KIND OF BLOOD	Q_{O_2} at 20°C.			Q_{O_2} at 37°C.			Q_{10} (27–37°C.)
	N	Mean	S.E.	N	Mean	S.E.	
Human				5	0.020	0.008	
Rabbit	3	0.023	0.004	3	0.037	0.007	1.37
Chicken	6	0.050	0.003	6	0.136	0.007	1.60
Frog	8	0.068	0.005	9	0.139	0.047	1.43
Fish	5	0.067	0.008	6	0.141	0.009	1.45
Sting ray (D. sp.)	3	0.127	0.003	3	0.266	0.003	1.45
Sting ray (D. centroura)				10	0.130	0.017	
Sting ray (D. centroura)				9[1]	0.184	0.020	

[1] Urea in the isotonic suspending medium.

It is interesting to note that the Q_{10}'s for all of the erythrocytes are of the same order of magnitude. This is what one would predict on the assumption of a similar series of chemical reactions in each type of erythrocyte.

The malic oxidase system

Malic dehydrogenase and the associated hydrogen carriers, DPN, cytochrome reductase, and the cytochrome oxidase system constitute the "malic oxidase" system. The overall activity of this complex of enzymes was assayed as follows (modified from Potter, '46):

0.1 ml of 20% potassium hydroxide in the center well
0.3 ml of 0.5 M potassium malate in the side arm
0.3 ml of 0.06 M DPN
0.3 ml of 2.0 M niacin
0.3 ml of 1×10^{-4} M cytochrome c
0.3 ml of 0.5 M potassium glutamate
1.4 ml of homogenate

The niacin is included to inhibit the enzymatic breakdown of the DPN (Mann and Quastel, '41). The glutamate serves to transaminate the oxaloacetic acid formed from the malate (Straub, '41) and thereby prevents product inhibition. This of course depends on the presence of transaminase in the homogenate.

Although in Potter's study the activity fell off very rapidly, this is not true with the erythrocyte homogenates as shown in figure 1 where the rate is constant for 40–50 minutes after tipping in the malate. The calculations of Q_{O_2} are based on 5 readings at 10 minute intervals after tipping. It is not feasible to add the DPN from the side-arm to initiate the reaction, since there is already considerable DPN in some of the erythrocyte homogenates (Leder and Handler, '51) and the reaction starts immediately on addition of the malate.

Of the nucleated erythrocytes, the chicken was studied in the most detail and figure 2 shows the linearity of the dilution curve over a wide range of homogenate concentration. In general about 100–200 mg dry weight of homogenate were used per vessel. This may appear to be a very concentrated preparation to use in the homogenate technique but it should be remembered that of the dry weight of the nucleated red cell 80–85% is inert hemoglobin and for the non-nucleated cell this value is about 95% (Ponder, '48).

The data in table 2 show that the malic oxidase activity of the homogenates is higher in chicken, fish and one species of sting-ray than in human and rabbit.

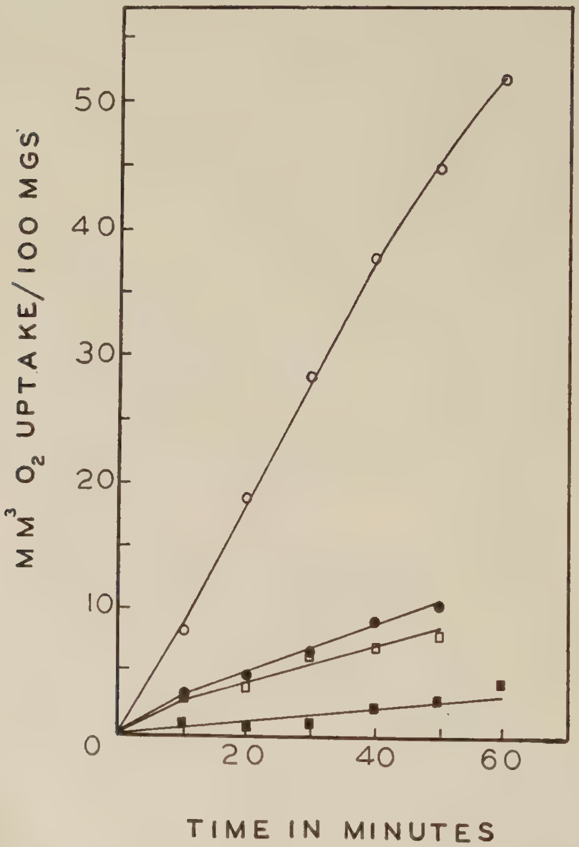

Fig. 1 Malic oxidase activity of erythrocyte homogenates
○ chicken, in the presence of 0.05 M sodium malate;
● chicken, no malate added;
□ human, in the presence of 0.05 M sodium malate;
■ human, no malate added.

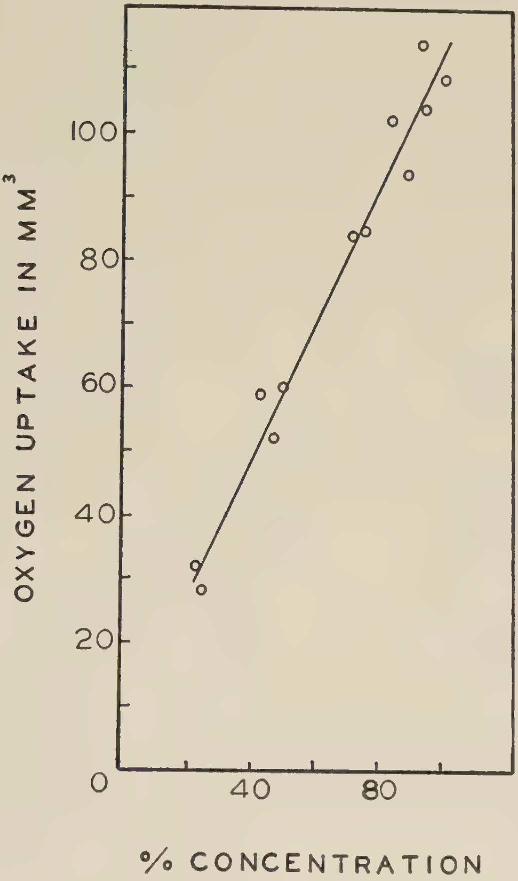

Fig. 2 Dilution curve of malic oxidase activity of chicken erythrocyte homogenates. One hundred per cent homogenate contains 0.2100 gm dry weight. Values are for 60 minutes (calculated on the rate for the first 50 minutes), obtained from three different homogenates.

TABLE 2

Malic oxidase activity expressed as Q_{O_2}

KIND OF BLOOD	NUMBER OF SAMPLES	MEAN	S.E.
Human	5	0.057	0.010
Rabbit	10	0.167	0.034
Chicken	14	0.544	0.028
Frog	5	0.104	0.015
Fish	8	0.313	0.020
Sting ray (D. sp.)	5	0.745	0.042
Sting ray (D. centroura)	5	0.178	0.040

The succinic oxidase system

Succinic dehydrogenase, the "Slater Factor" and the cytochrome oxidase system constitute the succinoxidase system. The technique of assay used is that described by Schneider and Potter ('43). The course of the reaction for a chicken and a human erythrocyte homogenate is shown in figure 3

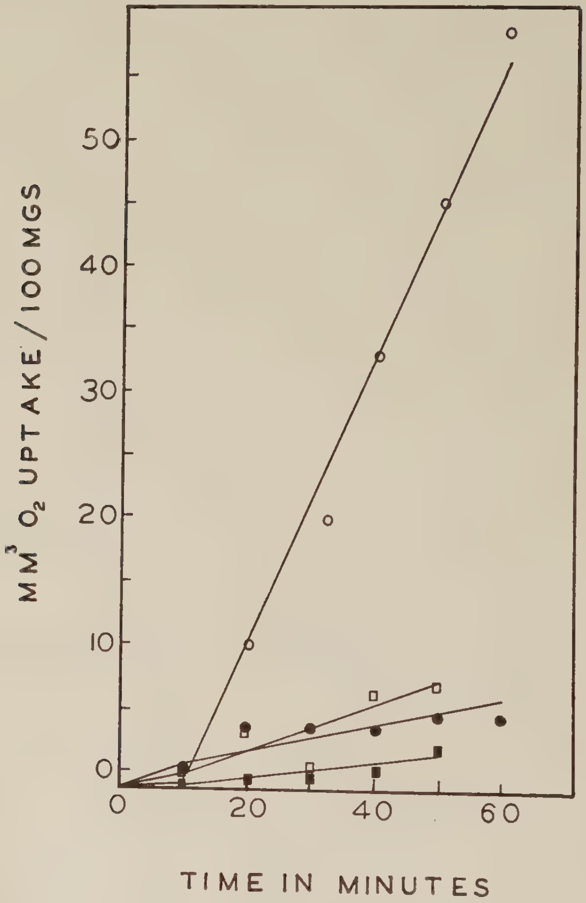

Fig. 3 Succinoxidase activity of erythrocyte homogenates
○ chicken, in the presence of 0.05 M sodium succinate;
● chicken, no succinate added;
☐ human, in the presence of 0.05 M sodium succinate;
■ human, no succinate added.

and it can be seen that the rate of oxygen consumption is linear for at least 60 minutes. The relationship between homogenate concentration and enzyme activity for chicken erythrocytes is shown in figure 4 and is linear. The Q_{O_2} values presented in table 3 were determined from 6 readings

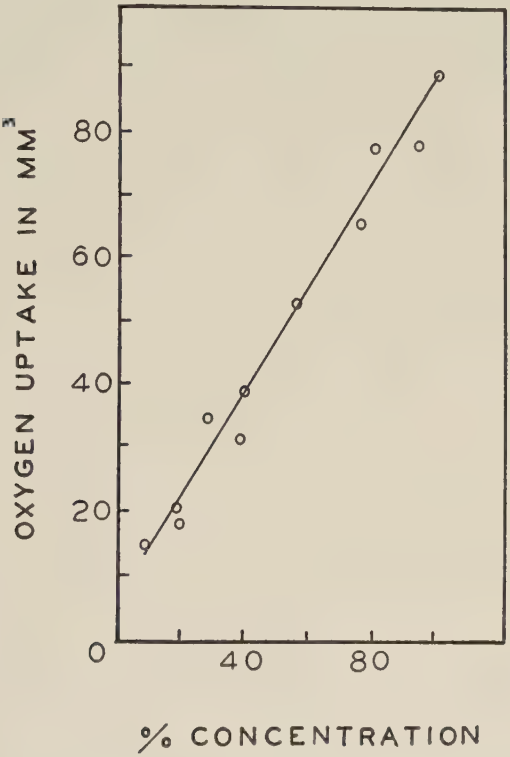

Fig. 4 Dilution curve of succinoxidase activity of chicken erythrocyte homogenates. One hundred per cent homogenate contains 0.1409 gm dry weight. Values are for 60 minutes, obtained from three different homogenates.

at 10 minute intervals after tipping. These data show that except for chicken erythrocytes there is very little succinoxidase activity in either nucleated or non-nucleated red cells. To test whether this actually represented succinoxidase activity, experiments were performed using two-side arm vessels with malonate in the second side arm. Addition of this substance completely inhibited the reaction thus validating the assay.

The initial experiments in this series were performed on homogenates of human and chicken erythrocytes (Baker and Hunter, '52). These preliminary data suggested that both the malic and succinoxidase systems had low activity in the non-nucleated erythrocyte but considerably higher activity in the nucleated cells. The subsequent data, however, do not allow one to generalize in this regard.

In assaying any "system" such as these two which have just been described, it is necessary to determine which individual step is limiting the overall rate of the reaction. It is usually assumed that cytochrome oxidase is present in homogenates in sufficient excess so that it is not rate-limiting. In most tissues studied this seems to be the case so that the activity of the succinoxidase and malic oxidase systems is a measure of succinic and malic dehydrogenase respectively. On theoretical grounds, one might postulate that a cell, such as a mammalian erythrocyte, which has a low rate of respiration might also have low cytochrome oxidase activity. Consequently, an attempt was made to assay for cytochrome oxidase in these erythrocytes.

TABLE 3

Succinoxidase activity expressed as Q_{O_2}

KIND OF BLOOD	N	MEAN	S.E.
Human	4	0.040	0.014
Rabbit	13	0.151	0.027
Chicken	21	0.534	0.031
Frog	5	0.119	0.051
Fish	8	0.033	0.019
Sting ray (D. sp.)	5	0.166	0.041
Sting ray (D. centroura)	13	0.151	0.027

The usual assay procedure includes the use of ascorbic acid for a substrate, and cytochrome c as an electron carrier. When this technique was tried with erythrocyte homogenates it was found to be unsatisfactory because the ascorbate reacted with the hemoglobin to form choleglobin. To date it has been impossible to obtain hemoglobin free homogenates with cytochrome oxidase activity.

Krebs cycle dehydrogenases

Since it was not possible to assay directly for cytochrome oxidase activity, an indirect approach was used. By sub-

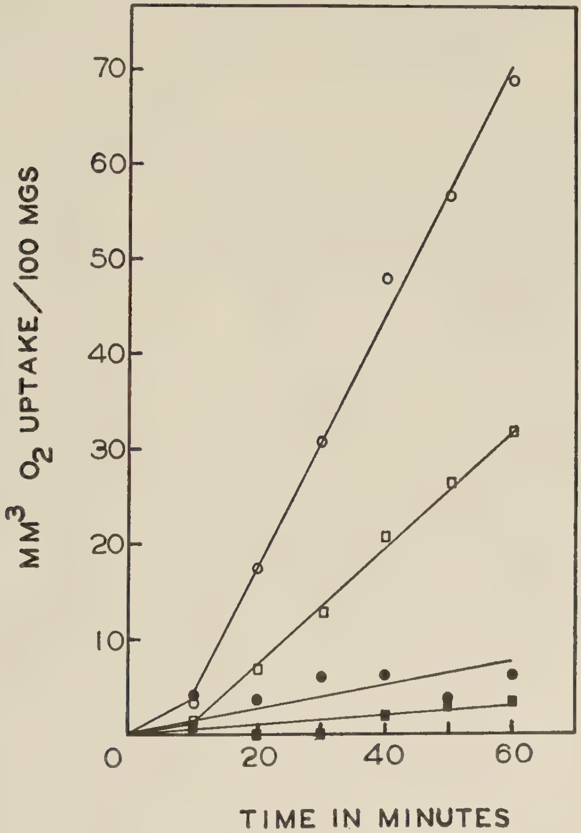

Fig. 5 Malic dehydrogenase activity of erythrocyte homogenates
○ chicken, in the presence of 0.05 M sodium malate;
● chicken, no malate added;
☐ human, in the presence of 0.05 M sodium malate;
■ human, no malate added.

stituting methylene blue as the terminal electron carrier, cytochrome oxidase was bypassed and the activity of the dehydrogenase could be determined directly. Malic dehydrogenase was assayed according to the system described above with the exception that 0.3 ml of a 1:10,000 solution of methylene blue was substituted for the cytochrome c.

It can be seen from the typical curves shown in figure 5 for the course of the reaction that the rate is constant for

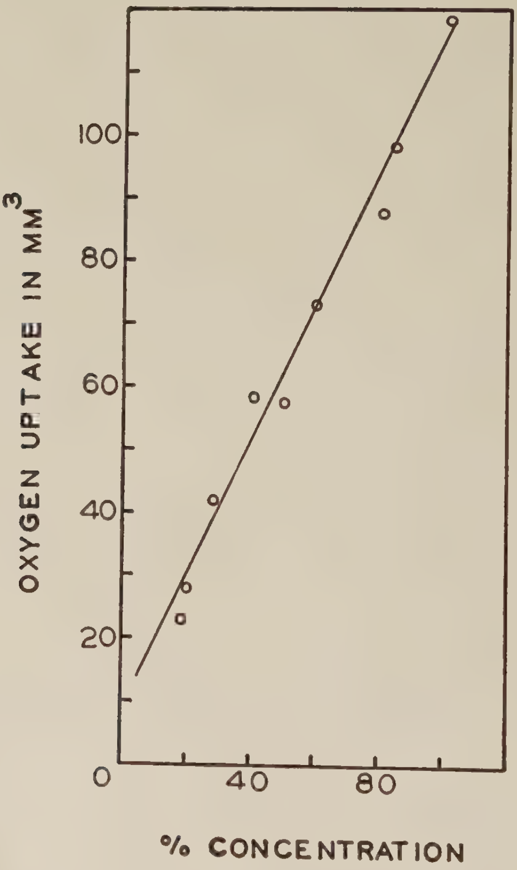

Fig. 6 Dilution curve of malic dehydrogenase activity of chicken erythrocyte homogenates. One hundred per cent homogenate contains 0.2048 gm dry weight. Values are for 60 minutes, obtained from three different homogenates.

60 minutes after tipping in the substrate. With chicken cell homogenates the clear-cut linear relationship between activity and homogenate concentration is shown in figure 6. Homogenates of rabbit cells, however, did not dilute linearly because of the presence of DPN-ase. Consequently it was necessary to use smaller amounts of homogenate (40 mg dry weight per vessel) and to use approximately the same amount of homogenate from one experiment to the next.

The data presented in table 4 show some malic dehydrogenase activity for all types of erythrocytes with no sharp differences between the nucleated and non-nucleated cells.

TABLE 4

Malic dehydrogenase activity expressed as Q_{O_2}

KIND OF BLOOD	N	MEAN	S.E.
Human	13	0.291	0.031
Rabbit	15	1.320	0.070
Chicken	12	0.709	0.032
Frog	5	0.370	0.043
Fish	10	0.770	0.063
Sting ray (D. sp.)	6	0.794	0.064
Sting ray (D. centroura)	9	0.374	0.045

Therefore it can be concluded that it is not the malic dehydrogenase which is responsible for the difference in malic oxidase activity shown in table 2. It is interesting to note that in all of the erythrocytes studied there is more malic dehydrogenase activity than there is malic oxidase activity. This comparison indicates that in the malic oxidase assay some hydrogen carrier with a more positive oxidation-reduction potential than methylene blue is limiting the rate of the reaction. If methylene blue picks up the electrons from diaphorase (Slater, '49), then cytochrome oxidase and/or the "Slater Factor" would be the rate-limiting step in these experiments. The difference between the malic oxidase and malic dehydrogenase activities is much greater in the mammalian than in the nucleated cells, which means that cytochrome oxidase (or the "Slater Factor") limits at a considerably lower level in the non-nucleated cells.

When a similar series of experiments was performed using methylene blue (or cresyl blue) to determine the activity of succinic dehydrogenase in chicken erythrocytes, no activity could be measured. The reason for this is not clear, and will be investigated further.

Lactic dehydrogenase

For a glycolytic enzyme, lactic dehydrogenase was chosen, and the following system, which is a modification of that described by Straub ('40) was used for assay:

0.05 ml of 0.002 M potassium hydroxide in the center well
0.05 ml of 2.0 M potassium cyanide in the center well
0.3 ml of 10% sodium lactate in the side-arm
0.3 ml of 0.06 M DPN
0.3 ml of 0.1 M niacin
0.3 ml of 1: 10,000 methylene blue
0.3 ml of 0.5 M potassium cyanide
1.4 ml of homogenate

The cyanide in this system inhibits further breakdown of the end product, pyruvic acid. It has been reported (Colowick, Kaplan and Ciotti, '51) that pyridine nucleotide reacts with cyanide and interferes with the spectrophotometric measurements, but at the pH used in most manometric experiments this reaction is negligible. Glutamate was tested for removing the pyruvic acid with chicken erythrocyte homogenates and was found to give similar values to those obtained using the cyanide. The activity, however, fell off sooner using glutamate (see fig. 7) and consequently only data obtained using the Cyanide technique are reported (fig. 8).

Figure 9 shows the linear relationship between the activity and concentration of chicken erythrocyte homogenates. The DPN-ase activity of homogenates of rabbit erythrocytes was so high that maximum values for lactic dehydrogenase could be obtained only by using very dilute homogenates. Erythrocytes of the other species studied did not have enough DPN-ase activity to interfere with the determinations.

In general the data in table 5 show a wide range of lactic dehydrogenase activity with no clear-cut differences between nucleated and non-nucleated erythrocytes.

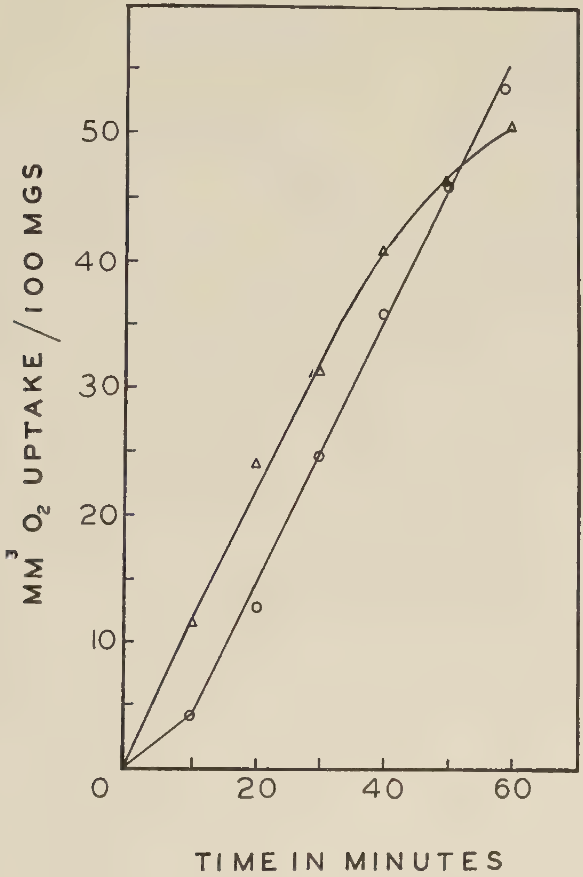

Fig. 7 Lactic dehydrogenase activity of chicken erythrocyte homogenates comparing the effectiveness of glutamate and cyanide in the assay.

△ chicken, in the presence of lactate and glutamate;
○ chicken, in the presence of lactate and cyanide.

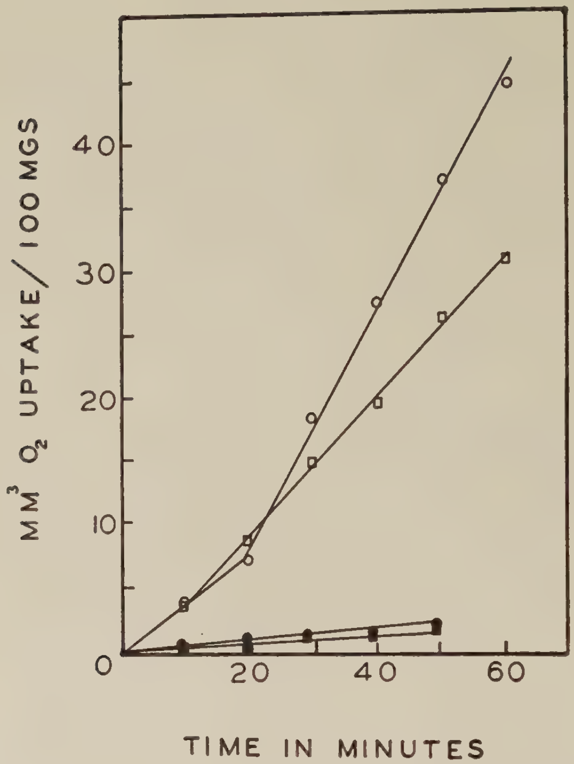

Fig. 8 Lactic dehydrogenase activity of erythrocyte homogenates

○ chicken, in the presence of 10% lactate;
● chicken, no lactate added;
□ human, in the presence of 10% lactate;
■ human, no lactate added.

TABLE 5

Lactic dehydrogenase activity expressed as Q_{O_2}

KIND OF BLOOD	N	MEAN	S.E.
Human	9	0.310	0.015
Rabbit	8	3.590	0.232
Chicken	13	0.531	0.035
Frog	5	0.440	0.100
Fish	13	0.441	0.046
Sting ray (D. sp.)	5	1.894	0.078
Sting ray (D. centroura)	5	1.016	0.102

DISCUSSION

The experiments measuring the rate of oxygen consumption of whole erythrocytes of various species definitely establish the fact that at the same temperature the nucleated erythrocytes of the lower vertebrates have a much higher activity than the non-nucleated mammalian red cells. These experiments were necessary since the values in the literature were obtained from experiments run at different temperatures.

The next step was to compare the activity of certain Krebs cycle and glycolytic enzymes in a nucleated and non-nucleated erythrocyte. A comparison was first made between the activities in homogenates of chicken and human red cells. The level of activity of malic, succinic and lactic dehydrogenase was relatively high in the chicken cells but only lactic dehydrogenase activity was found in the human cells. This led to the tentative suggestion that possibly the difference in levels of activity of the Krebs cycle enzymes in the two types of cells might account for the difference in respiratory rates (Baker and Hunter, '52). When erythrocytes of additional lower vertebrates were studied it was found that succinoxidase and malic oxidase activity was not uniformly high. Consequently, no generalization could be made concerning a difference in activities of Krebs cycle enzymes in nucleated and non-nucleated erythrocytes.

A number of interesting speculations suggest themselves from a further analysis of the data. If one compares the succinoxidase activity with whole cell respiration, only in rabbit and chicken cells is the Q_{O_2} for succinoxidase considerably larger than the Q_{O_2} for respiration. In fact, in frog cells and particularly in fish cells there is less of the former than the latter. Since it is usually assumed that the homogenate technique gives a value for the maximum activity of an enzyme system and since this is usually much higher than the regulated rate in the intact cell these data are unexpected. It might be that (a) the usual assay procedures were not satisfactory in the present experiments, (b) some alternate metabolic pathway functions in the nucleated cells, (c) some inhibitor of a portion of the succinoxidase system is present in some of the preparations, or (d) some portion of the succinoxidase system actually is rate limiting at a level which in turn limits the rate of respiration.

Several attempts were made to determine which of these alternative suggestions was correct. Since (d) seemed to be

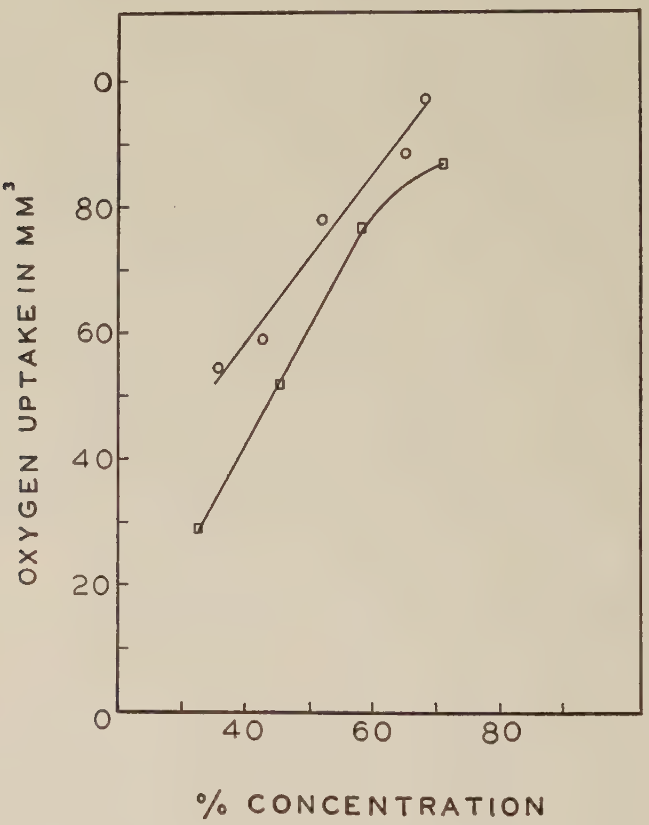

Fig. 9 Dilution curve of lactic dehydrogenase activity of erythrocyte homogenates.

○ chicken ☐ human

One hundred per cent chicken erythrocyte homogenate contains 0.2200 gm dry weight and 100% human erythrocyte homogenate contains 0.2210 gm dry weight. Each curve was obtained from a single homogenate.

the easiest to check, experiments were set up to measure the activity of succinic dehydrogenase and of cytochrome oxidase. In assaying for the activity of the succinoxidase system it is usually assumed that the enzymes terminal to the dehydrogenase are present in excess. The possibility exists, however, that in an erythrocyte where another iron porphyrin is present in such large amounts, there might be a limiting quantity of cytochrome oxidase (see, for example Duncan et al., '53). Unfortunately, we were not able to assay either cytochrome oxidase or succinic dehydrogenase. The ascorbate, which is used as a substrate in the assay of the former reacted with the hemoglobin in our preparations making it impossible to carry out the manometric determinations. Neither methylene blue nor cresyl blue acted as satisfactory electron acceptors in our attempts to measure the activity of the dehydrogenase.

Turning our attention to the data on the malic system proves more rewarding. A comparison of tables 2 and 4 shows that in every case except one type of sting ray the malic dehydrogenase activity is considerably greater than that of the malic oxidase system. In other words, when an electron carrier such as methylene blue is substituted for cytochrome c and cytochrome oxidase, considerably more oxygen is consumed. This would suggest that cytochrome oxidase is rate limiting in these cells. In the sting ray which had the highest rate of respiration (0.266) the ratio of malic dehydrogenase to malic oxidase is approximately 1. In man and in rabbit with the lowest respiratory rates (0.02 and 0.037 respectively) the ratios of dehydrogenase to oxidase are 5.1 and 7.9 respectively. The others with respiratory rates ranging from 0.130 to 0.141 have ratios between 1.3 and 3.6. These data would tend to substantiate the hypothesis that cytochrome oxidase is rate limiting in all these cells and in general, the lower the rate of respiration, the lower is the level of cytochrome oxidase activity.

Such a conclusion is different from that reached by Rubinstein and Denstedt ('53) following their analysis of tricarboxylic acid cycle enzymes in chicken erythrocytes. They state: "The ability of the avian erythrocyte to respire, in contrast to the mammalian red blood cell, appears to be due to the presence of a nucleus which contains many of the enzymes of the Krebs cycle." Our data on the two Krebs cycle enzymes in chicken erythrocytes certainly confirm their observations that these enzymes are present. There may be some question, however, about their localization in the nucleus. We have previously reported (Hunter and Baker, '53) that following freezing and thawing and centrifuging the enzymes in mammalian erythrocytes are present in the supernatant fluid while those of chicken red cells are present in the cellular debris. Microscopic observation of these "ghosts" shows that each nucleus is surrounded by a remnant of the cell membrane.

Although it is quite possible that the nuclei are responsible for the enzymatic activity of such preparations, we would prefer to reserve judgment until we have preparations in which the nuclei are more completely separated from the stroma. We have been unable to date to obtain such preparations from chicken erythrocytes. Preliminary experiments with erythrocytes of the sting ray suggested that in those cells, at least, a most interesting situation prevailed. Under certain experimental conditions it was possible to separate three fractions of these hemolyzed cells. There was no enzyme activity when the fractions were tested separately but recombination gave activity. These experiments suggested that in certain nucleated erythrocytes at least different components of some of the enzyme system were located on different portions of the cell. These could be separated by differential centrifugation. The whole problem of enzyme localization in erythrocytes needs clarification and experiments are being carried out in this laboratory in an attempt to do this.

In general our data compare qualitatively with those presented by Rubinstein and Denstedt. A detailed quantitative comparison is difficult, however, since different methods of assay were used in the two series of experiments. Usually the ferricyanide technique which they used gives higher values than the techniques we used (see, for example, Alivisatos and Denstedt, '51). Their value for succinoxidase for the whole hemolysate as shown in Table IV was $-Q_{O_2} = 0.13$ which is considerably lower than our figure of $-Q_{O_2} = 0.534$. The difference here may have resulted from the fact that they did not add cytochrome c whereas we did. Using their technique they were able to assay for succinic dehydrogenase. In their Table III they give a $Q_{O_2} = 0.91$ with an endogenous value of $Q_{CO_2} = 0.41$, in Table IV they give a Q_{CO_2} value of 0.49 for the whole homogenate. As they point out, the ratio of this Q_{CO_2} for the dehydrogenase to the C_{O_2} for the oxidase is close to the expected 4:1.

Another line of evidence which might suggest that low activity of cytochrome oxidase could not explain all of our observations comes from experiments in which respiratory rates of whole erythrocytes are measured in the presence of added substrates. Rubinstein and Denstedt include such data in Table II and we have done considerable work along this line particularly with rabbit reticulocytes (Hunter, Baker and Burr, '54). In some instances, added succinate increases whole cell respiration to a higher level than would be predicted on the basis of the amount of succinoxidase activity of homogenates.

It should be evident from this discussion that in our opinion considerably more work must be done before we can state with certainty the basis for the difference in metabolic activity of nucleated and non-nucleated erythrocytes. We do not believe that the suggestion made by Rubinstein and Denstedt can be reconciled with all our data. Some of our results are consistent with the suggestion that cytochrome oxidase is rate limiting in all erythrocytes studied and is limiting at a much lower level in mammalian than in nucleated red cells. Some of our other observations, however, might lead to the conclusion that using the standard methods for enzyme assays in this material leads to erroneous conclusions. It is hoped that additional experiments now being carried on in this and other laboratories may resolve this apparent paradox.

The theoretical implications of the determinations of the activity of lactic dehydrogenase are less complex. In general, all of the erythrocytes studied have a considerable amount of this enzyme present. This is what might have been predicted since there are no sharp differences in the glycolytic activity of these cells with the exception of the presence or absence of the Pasteur effect. One would certainly predict that both nucleated and non-nucleated red cells would have the enzymes necessary to form lactic acid. The determination of the absolute amount of lactic dehydrogenase present is complicated in some of these cells by the presence of large amounts of DPNase. This is particularly true of rabbit erythrocytes.

CONCLUSIONS

1. Measurements were made of oxygen consumption of whole cells and the activity of malic oxidase, succinoxidase, malic dehydrogenase and lactic dehydrogenase in homogenates of erythrocytes of sting ray, fish, frog, chicken, rabbit and human.
2. At a given temperature, the nucleated red blood cells of lower vertebrates respire at a level considerably above that of the non-nucleated, mammalian erythrocytes.
3. The succinoxidase activity of homogenates of chicken erythrocytes and the malic oxidase activity of homogenates of chicken, fish, and some sting rays are considerably higher than in other erythrocytes studied. The differences in activity cannot be correlated with the presence or absence of a nucleus.
4. There is considerable malic dehydrogenase activity in homogenates of all types of erythrocytes studied with no sharp differences between the nucleated and non-nucleated cells. There is more malic dehydrogenase than malic oxidase activity in all of the erythrocytes studied. This might be interpreted as meaning that cytochrome oxidase and/or the Slater Factor is rate limiting and limiting at a lower level in mammalian cells.

5. All of the erythrocytes studied had considerable lactic dehydrogenase activity with no consistent differences between nucleated and non-nucleated cells.

6. Certain possible interpretations of these data are discussed.

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EFFECTS OF pH ON THE HEART OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* LINNAEUS

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ONE FIGURE

Little work has been done on the effects of pH on the activity of isolated organs of insects such as the gut or dorsal blood vessel. Griffiths and Tauber ('43) studied the effects of pH values, which ranged from 6.8 to 8.9, on the activity of the crop of the American cockroach and found maximal activity at approximately 8.0. No work is available concerning the extremes of pH at which the activity of isolated living tissue can occur, or the changes in activity which accompany changes in the pH of the environment.

The purpose of the present experiments is to study the effects of changing the pH of a perfusing solution on the contractions of the isolated heart of the American cockroach.

MATERIAL AND METHODS

Isolated heart preparations were obtained from large nymphal or adult cockroaches by the method described by Yeager and Hager ('34). Previous to the dissection, the insects were anesthetized with CO_2. All observations were made over a 3-hour period with a binocular dissecting microscope (magnification, 36 ×) at regular intervals of 5 or of 10 minutes. The rates of contraction were calculated as number of beats per minute. Throughout the observation period, the hearts were perfused with a fresh supply of aerated saline solution maintained at 25°C. At each pH, determinations were made on at least 9 preparations.

Preliminary experiments were performed to determine the most satisfactory solution for the heart of the American cockroach. The following were tried: Yeager's, Carlson's, Lévy's, Ephrussi and Beadle's, Pringle's, Davenport's and Bêlâr's. The composition of each of these solutions may be obtained from the table given by Buck ('53). Locke's solution and those of Maloeuf ('35) and of Baber ('38) were also tried. Lévy's solution (9.0 gm NaCl, 0.7 gm KCl, and 0.46 gm $CaCl_2$ per liter) was found to be the most satisfactory and was used in all experiments. It was buffered (by the addition of 10 ml of buffer per liter) with Sörensen's glycine buffer mixture (Sörensen, '12). The solution was adjusted to pH values which ranged from 2.5 to 12.0 using increments of 0.5 of a pH unit. The pH readings were made with a glass electrode.

Lévy's solution with Sörensen's glycine buffer did not hold the pH around neutrality. Since the solution is aerated during each experiment, the effects of aeration on the pH of the neutral solution were determined. In these experiments, different amounts (1.0, 5.0, and 10.0 ml) of buffer per liter of solution were used, and the pH values determined after 15 or 30 minutes of aeration, and 15 minutes after the cessation of aeration. Various other buffers were used in an attempt to hold the pH at 7.0. These were Sörensen's ('12) borate and citrate (10 ml per liter), 0.01 and 0.03 M phosphate, 0.0004 M phosphate with borate or citrate, and 0.0004 M phosphate. In each case, the phosphates are listed as final concentrations.

OBSERVATIONS

Immediately after isolating the heart, there was a period of shock which persisted for varying lengths of time up to 1 hour. During this time the heart failed to beat. It was followed by a period during which the heart beat irregularly at first, but the rate changed gradually to a level characteristic of the pH.

At pH values of 2.5 and 3.0, disintegration was evident as indicated by points of rupture in the heart and the presence of tissue debris. At 3.5, the hearts beat for an average of 100 minutes; from 4.0 to 10.0, they beat for at least the 3-hour period. At higher values, the contracting heart gave the appearance of writhing or fibrillation and the rate of beat was difficult to determine. At 12.0, disintegration of cardiac tissue became evident after about 1 hour. In general acid solutions had a diastolic and alkaline solutions a systolic effect.

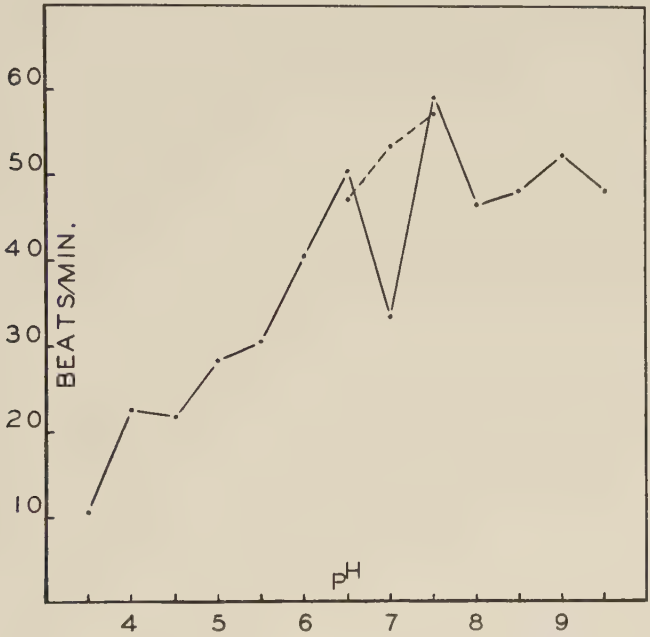

Fig. 1 Effects of pH on the rate of beat of the cockroach heart. Solid line, rate with Sörensen's glycine buffer; dotted line, rate with 0.0004 M phosphate buffer.

The rates of heart beat between pH values of 3.5 and 10.0 are plotted in figure 1. In general, the rate was proportional to pH up to the optimal value of 7.5. Above this pH, there was a slight decrease in rate. Figure 1 indicates that the hearts did not beat as rapidly at neutrality as they did at 6.5 and 7.5. It was found that at 7.0, aeration reduced the pH of Lévy's solution containing glycine buffer to approximately 5.5. Furthermore, allowing the neutral solution to flow over the heart resulted in changes in acidity. When the initial pH was 7.0, values as low as 5.4 were obtained after 15 minutes of perfusion. The value 5.6 occurred most frequently in a series of 10 readings. It may be noted in figure 1 that the rates plotted for 5.5 and 7.0 are comparable.

Experiments with other buffers showed that borate and citrate did not hold the pH around neutrality. Furthermore, the borate buffer, either alone or in combination with phosphate, was toxic. The concentrated phosphate buffers (0.01 and 0.03 M) precipitated calcium from solution. However, 0.0004 M phosphate was satisfactory around neutrality and was used at 6.5, 7.0, and 7.5. The results of these experiments are shown in figure 1 (dotted line). In each case, the pH was checked at intervals and was found to deviate less than 0.1 of a pH unit. The 0.0004 M phosphate buffer was unsatisfactory at 8.0. However, the glycine buffer held the pH approximately constant at this value.

DISCUSSION

The period of shock which followed the removal of the heart is comparable to the condition described by Roeder ('37) in his experiments involving the destruction of nervous tissue in the praying mantis, *Mantis religiosa*. In this insect, shock lasted from 5 to 30 minutes and was characterized by reflex activity which was unstable and variable. Roeder believed that it was the result of central stimulation during the operation. In the present experiments, shock may have been caused by severance of nerve connections, or by the inability of the heart to adapt itself immediately to the new environment.

The effects of acids and bases on the cockroach heart are the same as described for the vertebrate heart. In both instances, acids produce diastolic and bases systolic effects. However, the range tolerated by the cockroach heart is greater than that reported for vertebrate hearts. Dale and Thacker ('14) stated that the frog's heart is rather remarkably resistant to changes in the acid-base composition of its perfusate, and beats in solutions as acid as 5.5 and as alkaline as 10. With regard to the mammalian heart, Fulton (47) reported that the lethal pH in the intact anesthetized dog is very close to pH 6.0 (range 5.5 to 6.6).

The optimal pH of 7.5, obtained for the heart of the American cockroach, compares favorably with 8.0 found by Griffiths and Tauber ('43) for the crop activity of this insect.

SUMMARY

1. The heart of the American cockroach, perfused with Lévy's solution buffered with Sörensen's glycine buffer, was able to beat at pH values from 3.5 to 10.0. Below and above 12.0, tissue disintegration occurred.
2. Acids have a diastolic and alkalies a systolic effect.
3. Sörensen's glycine buffer was not able to maintain a constant pH around neutrality, the solution becoming more acid as a result of aeration and perfusion over the hearts. A constant pH was maintained near neutrality with Lévy's solution containing 0.0004 M phosphate buffer.
4. The optimal pH was found to be 7.5.

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